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THE BIOTHERAPEUTIC ACTION OF *TRYPANOSOMA CRUZI* ON TAR CARCINOMA IN MICE[1]

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From 1931 to 1947 Roskin and Klyueva and various collaborators published the results of a series of experiments on the therapeutic action of *Trypanosoma cruzi* and of the endotoxin prepared from it upon transplantable carcinoma and sarcoma in mice and subsequently on carcinoma in man. These results were quite favorable, and Malisoff has confirmed them (*1*). However, Hauschka, Saxe, and Blair (*2*) and Hauschka and Goodwin (*3*) repeated the procedures described by Roskin, Klyueva, and Malisoff as faithfully as possible but obtained negative results. Hauschka and Goodwin (*3*) reported that the sarcoma 180 substrain used by Malisoff regressed spontaneously in 55–60 percent of the untreated mice; consequently, Malisoff's experimental validation of Roskin's work is inadequately controlled. Hauschka *et al.* refrain from final evaluation of the work of Roskin because of contradictory results which they have obtained in using *T. cruzi* lysates from infected mouse plasma. However, they point out the possibility that infections other than Chagas' disease present in donor mice could affect the results. If this possibility proves to be valid, then *T. cruzi* is not the source of the active factor in the K. R.-endotoxin.

The experimental study presented here is designed to test the therapeutic or tumor-regression effect of *T. cruzi* and its endotoxin. The test object used is epidermal carcinoma of mice produced by skin painting with tar; this selection was made because it affords the possibility of both direct visualization and easy microscopic confirmation of the papilloma-

[1] Received for publication June 24, 1949.
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tous and carcinomatous growths from their beginning. Other reasons prompting this choice of test object were that there is no incalculable factor of spontaneous regression in tar-induced carcinomas and that the tumor itself is proper to the organism and to its tissue of origin.

The *T. cruzi* strain used in this study is the one isolated by Malamos from monkeys and received at the Leiden Parasitological Laboratory in 1936 from the Tropeninstitut of Hamburg. The endotoxin was prepared first by Roskin's technique, lysis of cultured trypanosomes by heating, and afterward by Malisoff's technique, lysis of the trypanosomes with pyrogen-free distilled water. One K. R. contains one million trypanosomes.

The standard procedure of painting the mice with tar twice weekly until the appearance of papillomata was utilized. At this point, treatment with endotoxin was begun. Also at this point, the tumors were photographed, and biopsied, and a blood smear was made. Following treatment, the mice were observed clinically, the tumors were measured weekly, and a blood smear was made. Efforts to control bacterial infections of the lesions were made by local treatment with sulfanilamide powder and sometimes with cibazol ointment. The mice were maintained on an adequate diet; supplemental vitamin B complex was added as the general condition of the mice indicated. After death the mice were autopsied; the lesions were measured, photographed, and examined microscopically; and a careful search for the presence of metastases was also made.

The experiments may be divided into 3 series: Series 1: 12 mice treated with the endotoxin prepared by Malisoff's technique, at first with a dosage of 1 K. R. per day, subsequently with a dosage of 10 K. R. per day. The endotoxin was injected as long as trypanosomes were available. Also included in this group are 2 mice which were injected for 5 days with the high dosage of 36 to 42 K. R. per day. Series 2: 5 mice treated with the same endotoxin for 8 days with a dosage of 1 K. R. per day. Series 3: 10 mice with the tumors of varying dimensions treated at first with intraperitoneal injections of mouse blood containing living trypanosomes and 3 weeks afterward (when the trypanosomes were present in the peripheral blood) with a dosage of 10 K. R. per day for 12 or 28 days.

Before discussing the results of these experiments, we should like to draw attention to two points made by Klyueva (*4*): The therapeutic effect of endotoxin on the tumor ceases when treatment is discontinued but returns immediately when treatment is recommenced. In those cases where a favorable reaction was observed, the therapeutic effect was constant regardless of the stage of the carcinoma.

In the course of the present experiment, intervals occasionally occurred between two treatments because of unsatisfactory preparation of the endotoxin. In those cases in which initial treatment produced a favorable reaction, the discontinuity of treatment is, therefore, probably not responsible for possible failures of subsequent treatments. The second point which Klyueva makes is important, because it was not technically feasible

in our experiments to have tar-induced carcinomas of a given dimension available as desired.

The results of the three experimental series are as follows: Series 1, high dosage of endotoxin (table 1): The injected endotoxin did not have a uniform effect on tumor growth without respect to dosage. In one case a single papilloma became smaller and remained small; however, tar-induced papillomata may sometimes regress spontaneously. Transition from papilloma into carcinoma could not be prevented by the treatment. Series 2, 1 K. R. of endotoxin per day for 8 days (table 2): The identical conclusions as formulated from table 1 are drawn. No effect was observed on mitotic activity. At autopsy one mouse showed metastases in both lungs and in the inguinal and preaortic lymph nodes. Series 3, living trypanosomes followed by 10 K. R. per day of endotoxin for 14 days (table 3): Small regression of the tumor surface was observed in 4 mice shortly before death; however, no other evidence of improvement was found. Microscopic examination revealed intracellular trypanosomes in the connective tissue and muscle around the tumors, in the stroma of the tumors, occasionally in tumor cells at the periphery of the tumors, and in 1 mouse a lung metastasis contained many cells infected with trypanosomes.

TABLE 1.—*Series 1*

Mouse No.	Nature of tumor in beginning of experiment (number of weeks old)		Treatment			Dimension of tumor in mm²					Result of autopsy: carcinoma
	Papilloma	Carcinoma	Number of units per day (during number of days)	Number of days between—		Before first treatment	After first treatment (autopsy)	After second treatment (autopsy)	Before third treatment	After third treatment (autopsy)	
				First and second treatment	Second and third treatment						
16	-------	+(8)	1 E (35) 10 E (31)	14	-----	99	-----	360	-----	-----	+
18	-------	+(8)	1 E (31)	-----	-----	25	314	-----	-----	-----	+
19	-------	+(4)	1 E (11) 10 E (13)	14	-----	100	-----	640	-----	-----	+
20	-------	+(3)	1 E (11) 10 E (43)	14	-----	25	-----	600	-----	-----	+
21	+(7)	-------	1 E (11) 10 E (47) 1 E (8)	14	35	36	4	1	49	300	+
22	+(14)	-------	1 E (11) 10 E (39)	14	-----	36	-----	300	-----	-----	+
23	+(8)	-------	1 E (11) 10 E (39)	14	-----	30	-----	325	-----	-----	+
24	-------	+(9)	10 E (19)	-----	-----	152	320	-----	-----	-----	[1] +
25	-------	+(9)	10 E (10)	-----	-----	344	900	-----	-----	-----	+
26	+(12)	-------	10 E (10)	-----	-----	130	450	-----	-----	-----	+
27	-------	+(9)	36 E (5)	-----	-----	190	480	-----	-----	-----	+
28	-------	+(9)	42 E (5)	-----	-----	190	440	-----	-----	-----	+

[1] Metastases in both axillary and inguinal lymph nodes.

TABLE 2.—*Series 2 (treatment with 1 E during 8 days)*

Mouse No.	Nature of tumor in beginning of experiment (number of weeks old)		Dimension of tumor in mm³		Result of autopsy: carcinoma
	Papilloma	Carcinoma	Before treatment	After treatment (autopsy)	
31	----------	+(17)	234	385	+
32	----------	+ (9)	40	250	+
33	----------	+(17)	265	315	+
34	+ (4)	----------	55	150	+
35	+(11)	----------	100	140	[1] +

[1] Metastases in lungs, inguinal and preaortic lymph nodes.

TABLE 3.—*Series 3 (Pretreatment with living trypanosomes)*

Mouse No.	Nature of tumor in beginning of experiment (number of weeks old)		Treatment			Dimension of tumor in mm³				Result of autopsy: carcinoma
	Papilloma	Carcinoma	Number of units per day during number of days	Number of days between— First and second treatment	Number of days between— Second and third treatment	Before first treatment	Before second treatment	Before third treatment	After treatment (autopsy)	
37	-----	+(15)	10 E (13)	------	------	145	160	380	[1] 315	[2] +
			10 E (13)	29	16	------	------	------	------	
38	-----	+(18)	10 E (14)	23	------	266	250	------	336	[3] +
39	-----	+(25)	10 E (14)	23	------	380	490	------	650	+
41	-----	+(28)	10 E (9)	23	------	575	580	------	[1] 560	+
43	-----	+(15)	10 E (3)	23	------	540	522	------	522	[3] +
44	-----	+(16)	10 E (14)	23	14	165	260	400	400	+
			10 E (13)	------	------	------	------	------	------	
47	-----	+(27)	10 E (10)	23	------	228	312	------	[1] 300	[4] +
48	-----	+(17)	10 E (9)	20	------	192	192	------	[1] 126	+
50	-----	+(17)	10 E (13)	20	------	135	340	------	460	+
51	-----	+(17)	10 E (13)	20	------	118	265	------	360	+

[1] Reduction of dimension of the tumor by shrivelling and necrosis.
[2] Metastases in lungs, rib, and diaphragm.
[3] Metastases in lung.
[4] Metastases in lung and inguinal lymph node.

DISCUSSION

The results obtained in these experiments are far from encouraging. One must, however, exercise a degree of caution in pronouncing a pessimistic judgment in the face of the more promising claims by other investigators. For example, we used only one strain of *T. cruzi*; it is possible that if a therapeutic action of the endotoxin exists, it may be inherent from only one specific strain or from strains which we did not utilize. Klyueva used a strain which was probably highly virulent, but, on the other hand, Hauschka and his associates prepared their endotoxin from the same strain and obtained negative results.

In contrast to the advantages in using tar-induced skin carcinoma as a test-object, it is a possible objection that the use of tar induces a state

of toxicity in the mice which makes them unsuitable to benefit from the possible therapeutic effect of *T. cruzi* endotoxin. However, by appropriate laboratory methods, we were able to maintain the mice long enough to establish whether or not such an effect existed.

Over and above such considerations, there is always the incalculable fact in biologic experiments that some extraneous influence is operating to produce favorable results in the hands of one group of investigators while similar procedures give only negative results in the hands of other investigators. It is never possible to duplicate the conditions of an experiment exactly. Such basic variables as food, climate, housing conditions, the ecliptic of the moon, *etc.*, may always be invoked as not meeting the original conditions.

Lastly, there is the possibility that tar-induced epidermoid carcinoma is not influenced by *T. cruzi* endotoxin whereas certain transplantable cancers are.

SUMMARY

The endotoxin of *Trypanosoma cruzi* (strain Leiden) does not inhibit the development and subsequent growth of epidermoid carcinoma in mice produced by skin painting with tar. This is irrespective of dosage or of previous infection with living *T. cruzi*. This finding is in essential agreement with that of Hauschka and his colleagues who failed to find encouraging results using both transplanted and spontaneous tumors.

The development of tar-induced carcinoma is still too poorly understood to enable us to speak of a growth-retarding action of the endotoxin. No striking example of it was observed in the present investigation. However, growth arrest was observed in four papillomas, even to the point of regression. Spontaneous regression has previously been observed in papillomas.

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INHIBITION OF MAMMARY-GLAND DEVELOPMENT AND MAMMARY-TUMOR FORMATION IN FEMALE C3H MICE FOLLOWING INGESTION OF THIOURACIL[1,2]

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INTRODUCTION

It is well recognized that a change of either genetic background or of milk agent or of hormonal secretions may influence the development of spontaneous mammary tumors in female mice. It has also been suggested that the inhibiting effect on the production of mammary tumors caused by dietary restriction, or by deficiency of specific nutrients, may be mediated through alterations in the hormonal secretions which influence mammary-gland growth (*1*, *2*, *3*).

The surgical removal of an endocrine gland will affect the secretion of other endocrine glands. It would be expected that a chemical agent capable of specifically blocking the secretion of a single endocrine gland would also alter the normal balance. Experiments were therefore initiated to create a prolonged hormonal imbalance by using such chemical agents as thiourea and thiouracil to interfere with the function of the thyroid gland.

Histologic and cytologic evidence has already been reported which indicates widespread effects on organs and tissues of mice that were probably mediated by such a hormonal imbalance (*4*, *5*, *6*). The expected hyperplasia and hypertrophy of the thyroid gland were accompanied by structural changes in other endocrine glands, notably the adrenal, pituitary and ovary. The atrophy of the ovaries and uterus was noteworthy. The mammary glands of adult mice that had ingested thiourea revealed considerable atrophy. The time of appearance of spontaneous mammary tumors was delayed but no decrease observed in the final tumor incidence.

It was thought, however, that a prolonged hormonal imbalance induced in the animal at an early age, before the mammary glands had achieved full development, might produce a more drastic effect on the architecture of the mammary glands, which in turn might produce a pronounced lowering of the incidence of mammary tumors. Thiouracil, having greater anti-thyroid potency and lower toxicity than thiourea, at least for the rat,

[1] Presented in part before the Am. Assoc. Cancer Res. Atlantic City April 1946.

[2] Received for publication July 27, 1949.

was used in the present study with particular attention directed to the effect of the antagonist on body weight, food intake, estrous activity, thyroid weight, mammary gland development, and genesis of mammary tumors.

MATERIALS AND METHODS

High mammary-tumor strain C3H virgin female mice, obtained from the National Cancer Institute colony, were used. Animals were individually housed in wire-cloth bottom cages. Food and tap water were available at all times.

Litter mate animals were divided into control and treated groups of 70 and 73 mice, respectively. An attempt was also made to pair the animals of each litter according to the length and regularity of the estrous cycle which had been determined during a 3-week pre-experimental period. At 9–10 weeks of age one group was placed on the control diet and the other on the experimental diet.

The control diet, No. 77, was composed of natural food stuffs (*7*). The experimental animals were fed the same diet to which had been added, 3.75 gm. of 2-thiouracil[3] per kilogram, No. 145. After 26 weeks, the amount of thiouracil was increased to 5.0 gm. per kilogram because estrous cycles were reappearing in many animals.

The body weight and food intake were measured at weekly intervals. Vaginal smears were taken 6 days weekly. Smears were taken by the lavage method, and the ratio of cornified cells to epithelial cells and leukocytes was determined in the wet, unstained condition. Whole mounts of the mammary glands were prepared, using the technique described previously (*4*). The size of hyperplastic nodules in whole mounts of mammary glands was measured with an ocular micrometer, using the largest diameter of the nodule. When the animals reached 5 months of age they were palpated once each week for mammary tumors. Animals were killed when death appeared imminent or when mammary tumors attained a maximum diameter of 1–1.5 cm., except for 9 mice in each group which were killed in groups of three during the 8th, 11th, and 17th weeks of the experiment. Thyroid glands were weighed on a microtorsion type balance immediately after removal from the animal.

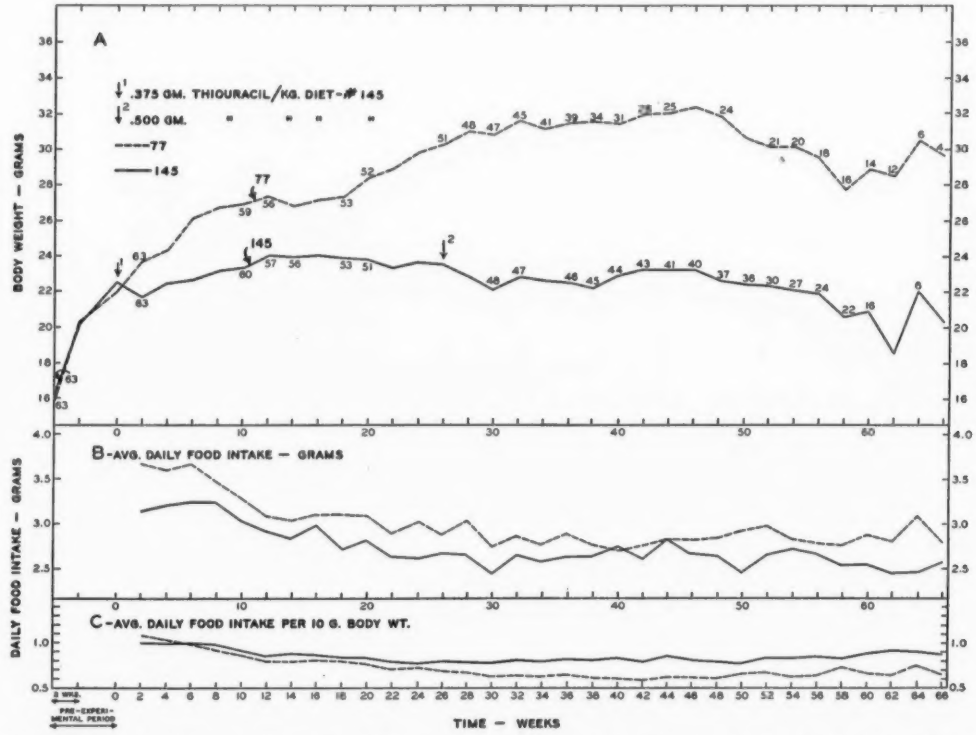
EXPERIMENTAL RESULTS

Body Weight and Food Intake

The body weight and food intake studies were based on 63 animals in each group because 7 control and 10 treated animals died during the first 6 weeks of the experiment and were excluded.

Body weight.—The body weights of the two groups of animals ingesting stock diet 77 were observed for a 5-week period previous to the start of the experiment. The growth of the two groups, as indicated by average body weight, was virtually identical during this pre-experimental period (text-fig. 1*A*).

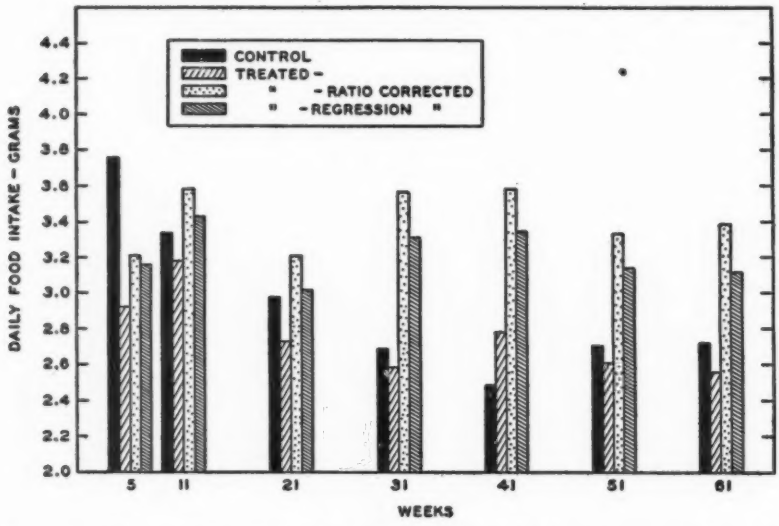
[3] 2-Thiouracil was generously supplied by Lederle Laboratories, Pearl River, N. Y., through the courtesy of Dr. S. M. Hardy.



TEXT-FIGURE 1*A*.—Average body weight of control and thiouracil-treated mice. *B*.—Average daily food intake of control and thiouracil-treated mice. *C*.—Average daily food consumption per 10 grams of body weight of control and thiouracil-treated mice.

Thiouracil was added to the diet of the experimental group when the mice were 9 to 10 weeks of age, the control animals being continued on stock diet No. 77. The control and treated animals continued to gain weight for 32 and 12 weeks respectively and the control group averaged approximately 3 grams more for the first 15 weeks, and thereafter approximately 9 grams more. The slight drop in body weight of the treated animals observed during the 26th week, occurred after increasing the level of dietary thiouracil (text-fig. 1*A*).

Food intake.—A comparison of the food consumption curves of the two groups (text-fig. 1*B*) indicated that the control animals ingested somewhat



TEXT-FIGURE 2.—A comparison of the ratio corrected and linear regression food intakes with the actual food intakes of thiouracil ingesting and control mice.

more food per day than did the thiouracil treated animals, the daily difference being 0.2 to 0.4 gram.

The situation is reversed when the food intake is considered on a unit of body weight. For each 10 grams of body weight the experimental mice ingested less food during the first 3 weeks but thereafter more food daily than did the control (text-fig. 1*C*).

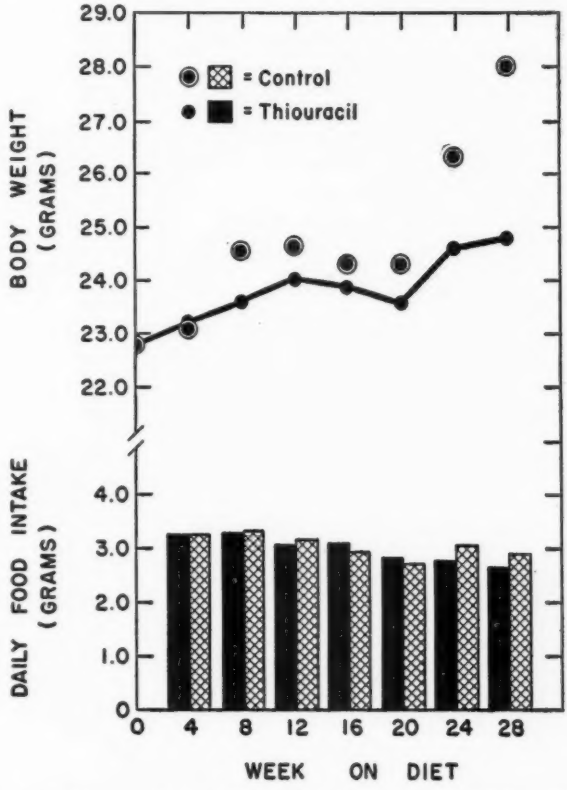
The food intake of the control and treated groups was compared further by obtaining: a ratio-adjusted food-intake value, and a linear regression food-intake value.[4] The ratio-adjusted food-intake values were calculated by the following formula:

$$\frac{\text{Average food intake}}{\text{Average body weight}} \text{ of treated mice} \times \text{average body weight of control mice.}$$

[4] The assistance of Jerome Cornfield, Office of the Statistical Coordinators, Division of Public Health Methods, in preparing the statistics is gratefully acknowledged.

Both types of estimations indicated that the food intake of the treated group was slightly lower than the actual control food intake for the 5th week, but thereafter the food intake of the treated group was always more than that of the control group (text-fig. 2, col. 3 and 4).

To determine whether food intake and body weight were related to estrous activity, the 10 treated mice whose estrous cycles had been studied continuously were compared with 5 control mice of similar body weight. The comparison was limited to the first 28 weeks, because during this



TEXT-FIGURE 3.—A comparison of the food intake of thiouracil-ingesting mice with control mice of comparable body weight.

interval the treated animals showed complete cessation and almost complete recovery of estrous activity.

The food intake of this selected control group during the 4th, 8th, and 12th weeks, exceeded the treated by 2 percent; during the 16th and 20th weeks, the treated food intake exceeded the control by 5 percent; while during the 24th and 28th weeks, the control intake exceeded the treated by 9 percent (text-fig. 3).

Estrous Cycles

The vaginal smears of all animals were observed for 3 weeks prior to starting the experiment. For the first 5 experimental months smears were taken of 10 animals, approximately 15 percent of each group. Beginning with the 6th month smears were continued on the same number of control animals and in addition all the treated animals were smeared.

The estrous response of the treated mice varied markedly from month to month so that a definite pattern of behavior became evident only when the observations were tabulated and summarized by monthly intervals. The data were analyzed in two ways: (1) Incidence or the percentage of animals per month showing vaginal cornification. (2) Frequency or the number of times per month a sharp rise in cornified cells occurred. No animal ever exhibited a normal estrous pattern during the continued ingestion of thiouracil. Complete cornification occurred at irregular periods, at other periods there were sudden sharp rises in the number of cornified cells accompanied by a scattering of leukocytes; both these conditions in the treated animal will be referred to as estrus.

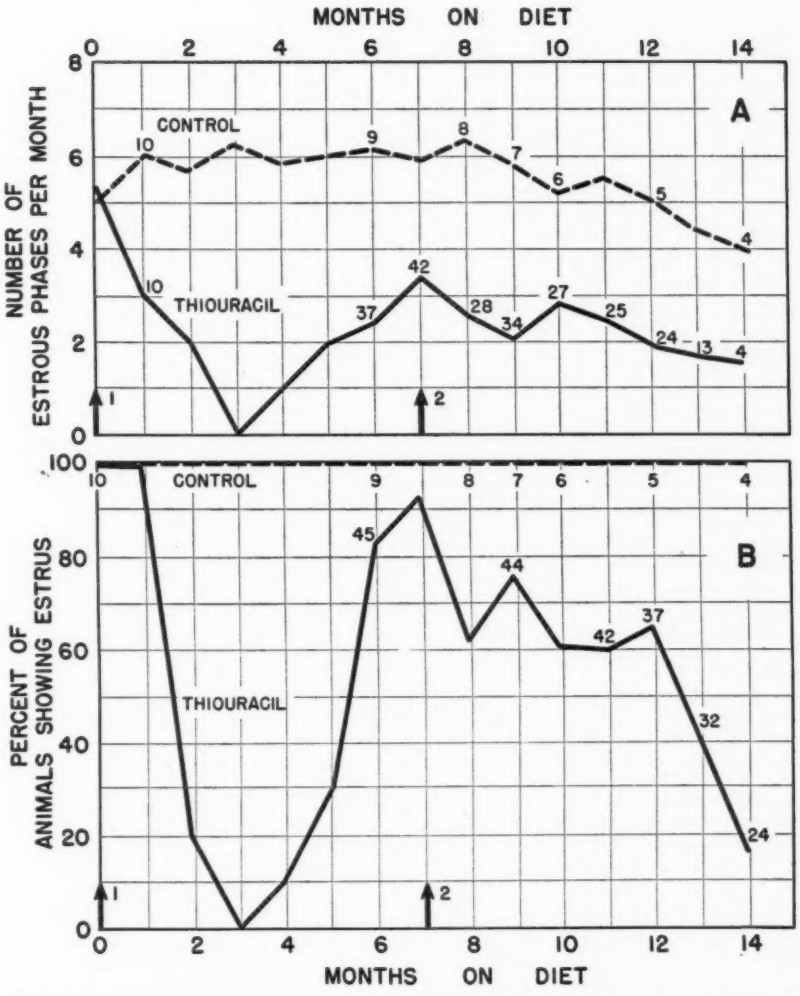
Estrous incidence.—All animals in both groups showed periods of complete cornification during the pre-experimental period (text-fig. 4*B*). The control group maintained this condition throughout the first 14 months of the experiment or until the animals reached 16 months of age. Ten remaining control animals observed during the 13th and 14th months also showed periods of estrus.

Vaginal cornification ceased from 4 to 28 days after initiation of thiouracil treatment (text-fig. 4*B*). Anestrus was maintained for the following 3 months with the exception of 3 animals, each of which displayed a single sudden sharp rise in cornified cells accompanied by numerous leukocytes. The incidence of vaginal cornification in the entire treated group rose to 93 percent during the 7th month, but one month later, after the dietary thiouracil level had been raised, the cornification incidence decreased to 62 percent and subsequently decreased even more.

Estrous frequency.—Estrous frequency followed a pattern similar to estrous incidence. During the pre-experimental period all animals passed through periodic intervals of complete cornification, the control and experimental groups averaging 5.0 and 5.3 estrous phases per month, respectively (text-fig. 4*A*). Both values are somewhat low because vaginal smearing was started when the animals were approximately 6 weeks old, and although some animals did not come into estrus until 5 to 10 days later, this initial period was included in the calculations.

Throughout the first 9 months of the experiment, the control animals were in estrus approximately 6 times per month; thereafter, estrous frequency dropped somewhat. The average number of estrous cycles of the ten remaining tumor-free control mice, during the 13th and 14th months, was very close to the value shown in the curve (text-fig. 4*A*). During this interval no difference in estrous behavior could be discerned between those animals which later developed tumors and those which did not.

Estrous frequency in the experimental group dropped immediately



TEXT-FIGURE 4*A*.—Frequency of vaginal cornification in control and thiouracil-treated mice. *B*.—Incidence of vaginal cornification in control and thiouracil-treated mice.

145=Thiouracil diet, 77=control diet
1=0.375 gm. thiouracil per kg. of diet
2=0.5 gm. thiouracil per kg. of diet
(Number above each point represents number of animals used to obtain value.)

after thiouracil ingestion, reaching zero by the third month. Following this initial anestrus there was a gradual increase in estrous frequency. Anestrous periods of from 6 to 9 weeks occurred in some animals preceded and followed by 1 to 3 irregular cycles. The peak in estrous frequency of 3.4 phases was reached during the 7th month, but following the increase

of the dietary thiouracil level, the average monthly estrous frequency dropped to two. Although estrous frequency in the control group also began to decrease after the 9th month, these animals never went below an average of 4 estrous phases per mouth, in contrast to the treated animals which never rose above an average of 3.4 estrous phases per month.

Mammary Glands

Gross mounts of mammary glands of 28 control and 58 experimental mice were examined. The age of the animals used ranged from 119 to 651 days, while the duration of thiouracil ingestion was 56 to 588 days.

Because of the dissimilarity in age and length of treatment, the architectural patterns of the mammary glands presented considerable variation, ranging from juvenile, relatively simple undeveloped glands, to adult, complex glands. A system of grading to describe the structural changes taking place was therefore adopted.

The criteria and structures used in this grading scheme were: (1) Acinar structures—location and number; (2) ducts—diameter-size and complexity of branching. The presence or absence of hyperplastic nodules (areas of alveolar hyperplasia) was not considered in this grading system. Three successive stages of development were selected:

Stage A—Simple: buds small and scattered with a few present in all regions of the gland; branching simple, with few side branches (figs. 1, 2, and 3).

Stage B—Intermediate: buds numerous and rounded; branching of ducts more complex with some smaller caliber ducts present (figs. 4, 5, and 6).

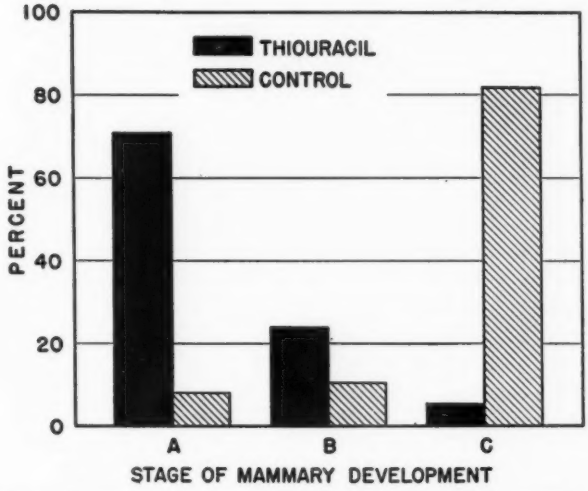
Stage C—Complex: buds profuse; branching ducts form an intricate pattern with very numerous small caliber ducts of varying diameters (figs. 7, 8, and 9).

A gland possessing a very simple structural pattern was graded stage A regardless of whether that pattern was due to the early age when killed or to the inhibition of development in an older treated animal. The more advanced stage of development was chosen, when the glandular structure was intermediate between two stages, in order to minimize rather than emphasize any inhibition of development which might be produced by thiouracil.

Extent of mammary development and length of treatment.—It was readily apparent when the animals had been grouped according to the stage of development each attained, that the majority of control animals (82 percent) achieved complex development while the majority of the treated animals (71 percent) never progressed beyond the most simple type of mammary architecture (text-fig. 5). Intermediate type of development was achieved by 11 percent of the control and 24 percent of the treated animals.

The comparison of mammary gland growth and length of treatment revealed that during the first 150 days of the experiment approximately half the control animals attained adult Stage C development. All control animals killed after this period showed adult development (table 1).

The relationship between age and length of treatment in the treated animals is not so well defined (table 1). Thus, after 50 to 250 days of



TEXT-FIGURE 5.—Percentage of control and thiouracil-treated animals possessing simple, intermediate, and complex stages of mammary development.
A=simple development
B=intermediate development
C=complex development

thiouracil administration the simplest degree of development, Stage A, predominated (fig. 2). After 250 to 399 days of treatment there was considerably more variation (figs. 6 and 9). After 400 days of treatment, the simplest type of development, Stage A, again predominated (fig. 3). In this treatment interval some animals continued to show a mammary architecture which was uniform throughout the gland as well as in glands from different sites, a condition similar to that found in animals killed during the earlier intervals of treatment. In other animals, however, mammary

TABLE 1.—*Stage of mammary development attained by mice on stock and thiouracil diets*

Days on diet	Number of mice presenting each stage of development					
	Diet 77 (control)			Diet 145 (thiouracil)		
	A	B	C	A	B	C
50– 99	2	1	3	6	0	1
100–149	0	2	1	4	1	0
150–199	0	0	2	1	0	0
200–249				2	0	0
250–299	0	0	2	1	2	0
300–349	0	0	2	4	3	1
350–399	0	0	3	6	5	1
400–449	0	0	6	13	2	0
450–499	0	0	3	2	0	0
500+	0	0	1	2	1	0
Total 50–500+	2	3	23	41	14	3

structure was more variable. In some mammae the basal portion of the gland was composed of numerous side branches and acini whereas the peripheral regions contained smoother ducts with less variation in duct diameters and fewer acini.

Hyperplastic Nodules

The whole mounts of all mammary glands were carefully examined for areas of alveolar hyperplasia or hyperplastic nodules. Because of the considerable amount of time involved in dissecting a mammary gland, all glands from each animal could not be mounted, but in the case of the thiouracil-treated animals, the stained portions of the glands which were not mounted were examined for nodules before discarding. In almost all cases portions of at least 2 or 3 mammae from the same animal were mounted and, occasionally, posterior as well as anterior glands were included. Nineteen of the 28 control and 46 of the 58 treated animals were killed after 150 days on the experiment and therefore had reached the age when hyperplastic nodules might be expected to develop in the mammary glands.

Incidence.—No hyperplastic areas were observed in any animals killed before they had been on the experiment 119 days. Nodules were initially observed in a control animal killed after 161 days. Since no animals were killed in the 120-to-161-day interval, there was no evidence from which to determine when hyperplastic nodules first began to appear. The glands of all 19 control animals killed after 161 days possessed one or more hyperplastic nodules.

A treated mouse killed after ingesting thiouracil for 280 days was the first one in this group which was observed as having a hyperplastic nodule. Although two treated animals bearing mammary tumors were killed previously, no areas of alveolar hyperplasia were observed in the remainder of the glands available for study. Fourteen of the forty-six treated animals which were killed after 150 days of thiouracil ingestion possessed hyperplastic nodules. The nodules were all located in one gland in 8 mice and nodules were found in more than one gland in the remaining 6 animals.

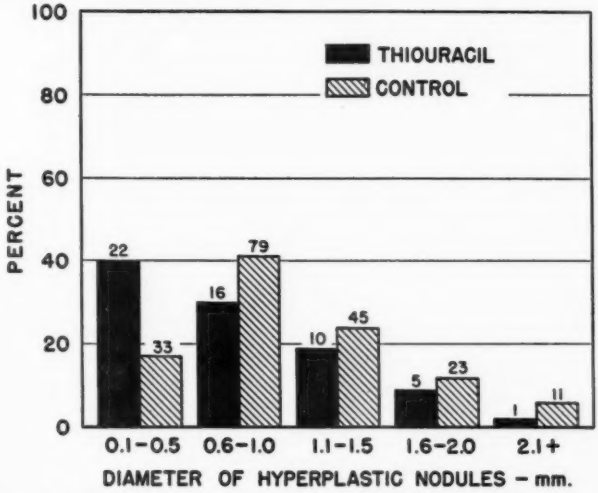
Number and size.—A reduction in the total number of hyperplastic nodules in the treated glands was also obtained. The glands from 19 controls had 191 nodules, an average of 10 per animal, while the glands from 14 treated animals had 54 nodules, an average of 3.8 per animal. If all areas of alveolar hyperplasia had been mounted for the control glands as had been done in the case of the treated animals, the control value would have been even higher.

A reduction in the size of the hyperplastic nodules was also observed. The average diameter was 1.0 and 0.79 mm. for the control and the treated groups, respectively. The range of diameter sizes emphasizes the difference between control and treated mice. Text-figure 6 shows the distribution of nodules according to size ranges. Seventeen percent of the nodules originating from the control group, and 40 percent of the nodules from the treated, were in the smallest size range. Nine hyper-

plastic nodules from the control group were 2.3–3.1 mm. in diameter, but the maximum diameter of the hyperplastic nodule from a treated mouse in this largest diameter range was only 2.1 mm.

In addition, it was found that the degree of mammary complexity and the occurrence of hyperplastic areas were directly related. All 19 control mice killed after 161 days had glands with complex, grade C development and all had hyperplastic nodules. Of the treated mice killed after 161 days, the 2 thiouracil mice with grade C development also had hyperplastic nodules, while only 5 of the 13 mice with grade B development and 6 of the 30 animals with grade A complexity developed hyperplastic nodules. The tendency therefore appears to be for hyperplastic areas to be found more frequently in mammae with complex architecture.

The paucity of acinar structures and hyperplastic nodules in the mammae is further illustrated by the fact that of the 10 treated mice



TEXT-FIGURE 6.—Percentage of control and treated hyperplastic nodules attaining a certain diameter range. The number in each bar represents the actual number of nodules in each diameter range.

developing tumors, only 4 seemed to possess hyperplastic nodules in the remaining mammae.

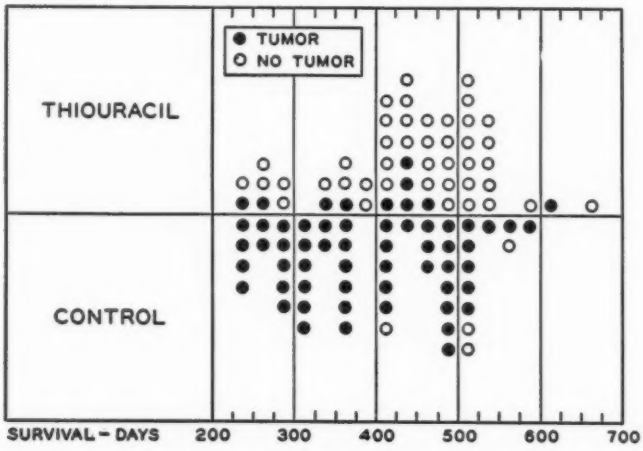
TUMOR INCIDENCE AND SURVIVAL

The tumor-incidence study was based on 53 control and 52 treated animals. Those animals which were sacrificed or died before 187 days of age were excluded.

Survival.—The effect of length of survival had to be considered before evaluating the tumor-incidence results, to make certain that adequate numbers of treated mice lived long enough to reach the age at which tumor formation should be expected. The average number of days of survival was 399 for the control and 436 for the treated mice. These two values are

not strictly comparable because most control animals developed tumors and were killed before the tumors became very large, while the majority of the treated animals failed to develop tumors and were allowed to live until death appeared imminent. Since the average survival time of the treated animals was at least as long as that of the controls, it was concluded that thiouracil ingestion did not affect the survival of the group too adversely.

The survival of individual animals in the two groups was next compared to determine how many animals died before they reached the age when tumors might be expected to appear. Five treated nontumor-bearing



TEXT-FIGURE 7.—Days of survival of control and thiouracil-treated mice. (Animals dying within a 25-day interval are grouped together.)

animals died before they reached the average age at tumor appearance of the control group and 10 treated nontumor-bearing animals died before they reached the average age at tumor appearance of the treated group (text-fig. 7). In addition, the average survival time of 436 days for the treated group was well beyond the average age at tumor appearance of both the control and treated groups. Thus it was concluded that adequate numbers of treated mice were available for the tumor-incidence study.

Tumor incidence.—A difference between the two groups was noted both in regard to incidence of tumors and age of the animal when the first tumor appeared. Mammary tumors were present in 49 control (92 percent), and in 10 treated (19 percent) animals. The range in ages at tumor appearance was from 187 to 529 days for the control and from 189 to 609 days for the treated group. The average age at tumor appearance was lower for the controls (330 days) than for the treated group (391 days) (table 2).

TABLE 2.—*Effect of thiouracil on age of appearance and incidence of mammary tumors*

Diet	Number of mice	Tumor incidence	Age when tumors appeared	Average age at tumor appearance
		Percent	*Days*	*Days*
77 (stock)	53	92	187–529	330
145 (thiouracil)	52	19	189–609	391

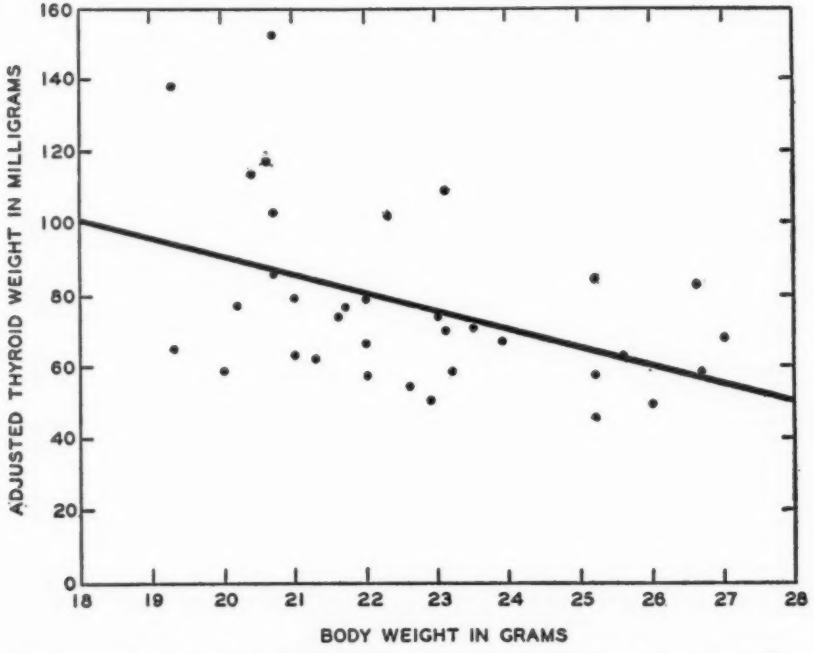
Tumor location.—Mammary tumors usually arise from both the anterior and posterior mammae. This was true for the control group, and the ratio of anterior to posterior tumors was 2:1.1. An unexpected difference occurred in the tumor locations of the thiouracil treated animals; all tumors were located in the region of the neck or shoulders, except for one in the axillary region.

THYROID-GLAND ENLARGEMENT

Changes were observed in many other tissues and organs of the thiouracil-treated mice, notably the decreased size of the ovaries, uteri, and adrenal glands, and the greatly increased size of the thyroid gland. Thyroid metastases to the lungs were also observed, but did not appear to originate from thyroid glands in any specific weight range, nor did their presence seem to alter any of the other systems studied. In addition, the pituitary cell population shifted, showing a decrease in alpha cells and an increase in beta cells. Although these changes were described in detail elsewhere (*6*), it was important to see whether any correlation existed between the results described herein and some of the changes mentioned above.

Thyroid-gland weight and body weight.—The tremendous enlargement of the thyroid gland and the early cessation of growth and low body weight which occurred with prolonged thiouracil ingestion raised the question of a possible correlation between thyroid weight and body weight. A statistical comparison was, therefore, made between thyroid weight and body weight. The body weight 60 days prior to death was selected because it was thought to represent more nearly the weight of a treated animal, since the animals frequently lost weight during the terminal weeks of life. Owing to a wide variation in the length of time thiouracil was ingested (264–588 days) by the 41 nontumor animals used, their thyroid weights were adjusted statistically to that of 400 days of treatment. Since there was so little correlation between body weight and length of treatment, this correction was practically without influence on the regression of thyroid weight on body weight and consequently was not made on the data presented. The distribution of the adjusted thyroid weight on body weight shows a definite inverse correlation (text-fig. 8) with a regression of 5.1 mg. thyroid weight per gram of body weight ($.01<P<.02$). Inclusion of tumor-bearing animals in the above comparison rendered the correlation insignificant because in general the mice with tumors had lower thyroid but similar body weights.

Thyroid-gland weight and mammary-gland development.—Particularly suggestive would be the existence of a relationship between thyroid-gland enlargement and mammary-gland growth and/or tumor genesis. Accordingly a statistical analysis was performed designed to answer two questions: Were thyroid-gland weights of animals with a minimum of mammary growth different from those possessing more complex development? Were thyroid-gland weights of tumor-bearing animals different



TEXT-FIGURE 8.—Distribution and regression of thyroid weight as a function of body weight in nontumor-bearing thiouracil-treated mice. The thyroid weights were adjusted to a mean of 400 days of thiouracil ingestion.

from those of nontumor-bearing animals (irrespective of the degree of mammary development)?

The general observation that thyroid-gland weight was proportional to length of treatment (*6*) necessitated an adjustment in thyroid-gland weights to correct for the influence of variations in the length of thiouracil ingestion, since this period ranged from 56 to 588 days. Consequently, the thyroid-gland weights of mice with Stage A mammae were adjusted to equal the same treatment interval as was represented by the thyroid glands from mice with Stage B mammae. A similar adjustment was made for nontumor- and tumor-bearing mice (table 3).

TABLE 3.—*Effect of thiouracil on average thyroid-gland weights of mice, grouped according to stage of mammary development and tumor genesis, and adjusted weights for variations in length of thiouracil ingestion*

	Number of mice	Period of thiouracil ingestion		Thyroid gland weight		Difference		Regression coefficient	Standard error of difference	Probability	
		Observed	Adjusted	Observed	Adjusted	Observed	Adjusted			Observed	Adjusted
		Average days	*Average days*	*Average mg.*	*Average mg.*	*Mg.*	*Mg.*				
Nontumor stage A mammary development	[1] 35	419	370	84. 4	[2] 76. 3	24. 9	16. 8	+0.1655	9. 3	[4] [6] <0. 01	[4] [6] <0.05
Nontumor stage B mammary development	[1] 10	370	370	59. 5	59. 5			. 1235			
Noncancerous	46	405	388	77. 1	[3] 74. 6	35. 8	33. 8	. 1487	4. 7	[5] [6] <. 001	[5] [6] <. 001
Cancerous	9	388	388	41. 3	41. 3			. 0706			

[1] One nontumor-bearing mouse classified as having stage C gland development was not included in these calculations but was included in the noncancerous group.

[2] Obtained by reducing 84.4 by (419–370)×0.1655. The regression coefficient, +0.1655, is the average daily increase in thyroid-gland weight for all animals with stage A development.

[3] Obtained by reducing 77.1 by (405–388)×0.1487. The regression coefficient, +0.1487, is the average daily increase in thyroid-gland weight for all noncancerous animals.

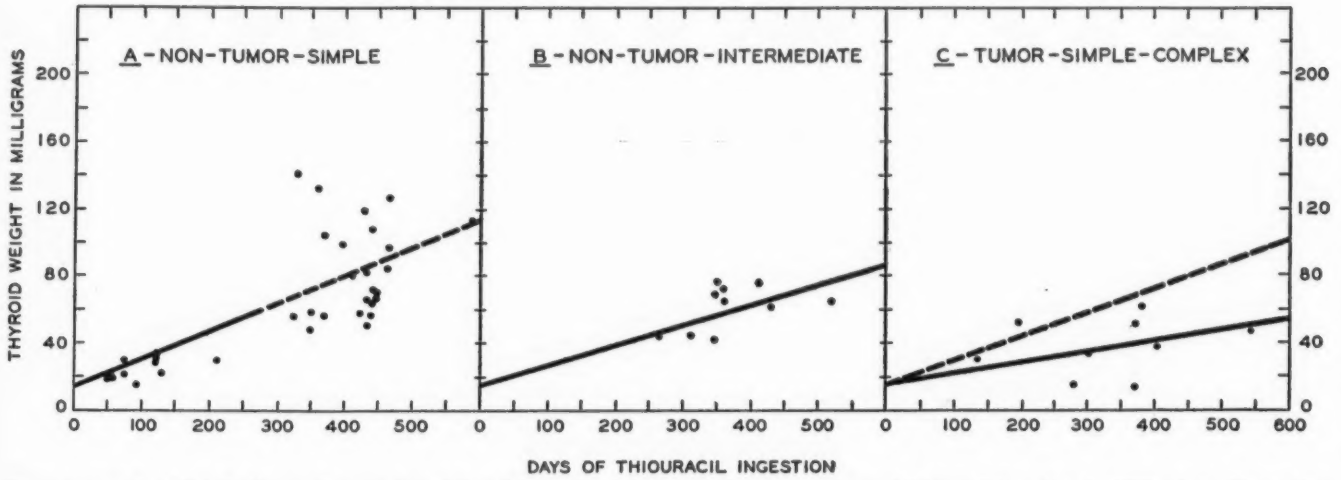
[4] This is the probability that by chance alone thyroid-gland weight in mice with stage A development would exceed the weight of the thyroid gland in mice with stage B development by the difference indicated.

[5] See footnote 4.

[6] The usual test for the significance of a mean difference is a test for the significance of the magnitude of the difference either positive or negative. The hypothesis advanced herein is that thyroid glands from mice with stage A mammary development would never weigh less than the thyroid glands from mice with stage B development (except by chance); a similar hypothesis was made for thyroid gland weights of nontumor- and tumor-bearing mice. The appropriate test of significance would then be to test how frequently a positive difference only of the magnitude observed could occur by chance. This is a common procedure in statistics and the one applied here.

As indicated in table 3, the average adjusted thyroid gland weight of 76.3 mg. for Stage A mice is significantly higher than the thyroid-gland weight of 59.5 mg. for mice with Stage B mammae. Likewise, the average adjusted thyroid-gland weight of 74.6 mg. for the noncancerous group is significantly higher than the 41.3 mg. for the cancerous group. The regression coefficient of +0.1655 for Stage A mammary development is significantly higher than +0.1235 for Stage B group ($0.05<P<0.01$). The regression coefficient of +0.1487 for nontumor-bearing animals is also significantly higher than for tumorous animals ($P=0.001$) (table 3 and text-fig. 9).

This analysis, therefore, indicates that the thyroid glands of animals with the simplest, Stage A, mammary development were larger and increased in weight at a more rapid rate than did the thyroid glands of animals with intermediate, Stage B development, and that animals without tumors possessed thyroid glands that were larger and increased in weight at a more rapid rate than did the thyroid glands of tumor-bearing animals. It is noteworthy that the thyroid glands of tumor-bearing animals were



TEXT-FIGURE 9.—Distribution and regression of thyroid-gland weights as a function of days of thiouracil ingestion in C3H mice with varying degrees of mammary development; and with and without tumors.

A. Thyroid-gland weights of nontumor-bearing mice with stage A mammary development.
B. Thyroid-gland weights of nontumor-bearing mice with stage B mammary development.
C. Thyroid-gland weights of tumor-bearing mice possessing all stages of mammary development.

(Solid lines represent calculated regression line of each group, dashed line represents the regression line of all nontumor animals.)

considerably smaller despite the fact that the mammae of these mice represented all stages of mammary complexity.

DISCUSSION

The results obtained will be discussed under 5 points: (1) body weight and food intake, (2) estrous cycles, (3) mammary-gland development, (4) mammary tumors, and (5) thyroid-gland weight. The results will be evaluated as to their relation to each other in (a) the individual animal, (b) the group as a whole. This will be followed by a reference to other regimens producing somewhat similar effects, and finally by a consideration of the possible mechanism involved in this experiment.

There was considerable variation in individual treated animals, and the correlation between body weight, estrous behavior, mammary growth, and mammary-tumor development was not always apparent. Thus, the heaviest animal did not always have the most regular cycles, nor did the animal with the most frequent estrous cycles have mammary glands that were most highly developed; similarly, mammary tumors did not always appear in animals which showed the highest degree of mammary-gland development.

These irregular responses of individual animals to ingested thiouracil seemed to point to an interaction in variable degree of several factors which finally resulted in a lowered incidence of mammary tumors. This trend was much more clearly defined and definite when the treated group was considered as a unit.

Body weight and food intake.—The smaller size of the thiouracil-treated animals, however, cannot be completely explained by the quantity of food ingested. The average daily food intake of the control group usually did not exceed that of the treated group by more than 0.1 to 0.3 gram. The treated animals consumed more food per unit of body weight than the controls and yet did not increase in weight, suggesting a possible interference with food utilization rather than toxicity of thiouracil.

This seemingly lowered efficiency of food utilization in mice is in sharp contrast to the higher efficiency of food utilization noted by Bratzler, *et al.* (*8*) in young male rats ingesting diets containing 0.1 and 0.2 percent thiouracil. The control and treated rats were fed the same quantity of food daily for 70 days. The treated rats increased more in body weight and body fat, and had a lowered heat production than the control animals. The body weight of mice treated with thiouracil, on the other hand, was less than their controls, and while no quantitative measurements were made of carcass composition, qualitatively it was noted that treated mice showed at autopsy less visible fat than controls. It is suggested that the higher level of thiouracil used in the mouse experiments may partially account for the difference. Such a view, also, seems to be supported by the rat data which showed that animals ingesting the largest amount of thiouracil gained less in protein than those ingesting the smaller amount.

Estrous cycles.—The gradual increase in estrous activity of the treated mice prior to raising the level of thiouracil suggests a shift in the

hormonal balance or a mounting tolerance to the drug. The response of the younger mice to thiouracil differed from the response of older mice receiving thiourea (*4*); these either maintained a permanent anestrus or continued to have estrous cycles at regular or irregular intervals. The differing patterns of response might be explained by such variables as the relative potencies of the two compounds, the initial level of goitrogen used, or the differing ages of the two groups.

Drasher and Zahl (*9*, *10*) noted that *Lithospermum* fed to C3H mice produced an immediate and persistent anestrus. The Rockland strain, however, responded to Lithospermum in a manner similar to the C3H response to thiouracil, an "anestrus followed by varying degrees of developed refractoriness to the drug as evidenced by the reappearance of normal estrous cycles."

It is noteworthy that during the first 28 weeks of the experiment estrous activity originally was at a maximum when thiouracil administration was initiated, then was completely suppressed and subsequently made an almost complete recovery (fig. 4). Yet during this same period the food consumption of the treated group showed little change and the difference between the food intakes of the control and treated groups did not exceed 9 percent.

Irregularity or absence of estrus has also been produced by a low-protein diet deficient in cystine (*11*). White and White (*12*) concluded, from indirect evidence, that estrus was not completely inhibited in animals maintained on a diet deficient in lysine.

Mammary glands.—The partial suppression of estrus indicates a possible disturbance of ovarian function, and inhibition of mammary gland development was to be expected. What was surprising was the lack of relationship between the degree of mammary development and estrous behavior. For example, a treated animal undergoing 2 to 5 estrous cycles per month over a 6-month interval might show no greater mammary development than one which had only 5 cycles during the same period. Even the treated animals which apparently possessed glands of adult, virgin complexity indistinguishable from the controls, displayed the same type of estrous behavior as the mice which showed extreme suppression of mammary development. Estrous activity under the conditions of this experiment then may serve as a slight indication of what is taking place in the mammary gland but cannot be used to predict the degree of mammary development; conversely, the extent of mammary development cannot be used as a measure of estrous activity.

The final mammary-gland picture is in part the result of the length of time the glands are exposed to hormonal stimulation. In the normal animal this period is roughly equivalent to the age of the animal, and during the growth span of the mammae, animals of increasing age possess glands of increasing complexity (*13*). The control animals in this experiment show such a direct relation between age and complexity of structure even though the mammae of all animals did not develop at the same rate (table 1).

In the thiouracil animals, hormonal stimulation of the mammary glands is apparently suppressed in varying degree, and the duration and extent of the suppression appear to some extent to be dependent upon the period of thiouracil ingestion. The mammary picture in the treated animal then is the result of the interaction between two processes; hormonal secretion and consequent stimulation of development, as opposed to suppression of hormonal secretion resulting in inhibition of development. As a result the thiouracil-treated animals at all age intervals failed to achieve complex mammary growth, and only three animals in the entire group reached the highest degree of complexity.

The predominance of the simplified type of mammary architecture can be interpreted as being due either to arrest of development or inhibition. The glands of older mice which ingested thiourea (*4*), showed a progressive pattern of inhibition proportional to the duration of treatment, beginning at the periphery of the gland and gradually encompassing the entire structure. In several instances the thiouracil-treated animals also possessed glands which showed greater complexity in the basal than in the peripheral portion. It is possible that if more animals had been killed previous to 400 days of thiouracil ingestion a larger number of Stage B and C glands would have been obtained. However, the lack of complexity found in animals killed during the earlier treatment intervals suggests that involution was not an important factor but rather that the underdevelopment was due mainly to arrested growth.

Mammary-gland development has also been influenced by underfeeding, calorie restriction, and amino-acid deficiencies. Myers (*14*) limited the body weights of young albino rats by restricting their food intake, and found that the retardation in development of the mammary gland was roughly proportional to the retardation in body weight. Huseby, *et al.* (*2*, *3*) described a similar underdevelopment of the mammae in mice which were subjected to a one-third restriction in calories. They considered the possibility that calorie restriction may lead indirectly to a protein deficiency and that the latter rather than the former might be the limiting factor. White (*15*) has shown that lysine or cystine deficient diets inhibited mammary development in mice.

Hyperplastic nodules were frequently absent in thiouracil-treated mice, and, when present, were generally fewer and smaller than nodules observed in control glands. This is consistent with the substantial underdevelopment of the mammary glands but contrasts with the apparent failure of thiourea (*4*) to eliminate hyperplastic nodules already present in the glands of adult animals. After further study, Huseby and Bittner (*16*) report that calorie restriction also was effective in reducing the number of hyperplastic nodules.

Mammary-tumor genesis.—The inhibition of mammary-tumor genesis is the logical sequel to the syndrome initiated by the action of thiouracil. Even though there was a striking reduction in tumor incidence, the average time of the appearance of tumors in the treated group was delayed

only 2 months, a delay of the same magnitude as was observed following thiourea ingestion (*4*).

Mammary-tumor genesis has been suppressed in varying degree by the same nutritional regimens which inhibit mammary-gland growth, supporting the view of a disturbance of the hormonal balance. These regimens include restriction of total food intake, restriction of calories (carbohydrates or carbohydrates and fats), diets low in protein and cystine or low lysine, and ariboflavinosis (*11*, *12*, *17*, *18*, *19*, *20*). The specific mechanisms by which nutritional deficiencies and goitrogens evoke their effect and their similarities or differences remain to be ascertained.

The observation on the difference in location of mammary tumors in the control and treated groups was unexpected. In an analysis of locations based on 795 tumors appearing in 649 stock and experimental animals of the C3H strain in which only the first tumor to arise was considered (multiple tumors were included only when they appeared to have arisen simultaneously), tumors were found to occur in both the anterior and posterior regions. The ratio of anterior to posterior tumors was 2.0:1.2;[5] the control tumors in this experiment had a similar ratio, 2.0:1.1. The restriction of mammary tumors to the anterior mammae in the treated mice, therefore, is somewhat puzzling.

A possible explanation for the difference in tumor incidence in the two locations is that mammary glands from specific sites may possess a differential capacity of response to hormonal stimulation. Lewis and Turner (*21*) report that following injection of mammogen to male mice, the anterior rudiments showed higher proportions of positive responses and more complex stages of development compared to the posterior rudiments. The small number of posterior glands studied in the thiouracil mice made it impossible to determine whether the anterior mammae were more complex than the posterior ones. Nevertheless, it is suggested that in the thiouracil experiment, the level of hormonal stimulation may have been sufficient, in some instances, to develop the anterior mammae to the stage permitting mammary tumor formation, but inadequate for equivalent development of the posterior mammae.

Thyroid-gland weight.—The hypertrophy and hyperplasia of the thyroid gland following administration of thiouracil are thought to be due to an increase in thyrotrophin secretion in response to the lowered thyroxin output (*22*).

Cramer and Horning (*23*) have reported that thyrotrophin inhibits mammary-gland development in estrogenized male mice, and also inhibits spontaneous mammary-tumor genesis in female mice, although later workers (*24*) were unable to reproduce Cramer and Horning's results. The supposedly antagonistic action of thyrotrophin on estrogen as suggested by Cramer and Horning could be responsible for the inhibition of mammary-tumor genesis in the thiouracil-treated mice. Such an explanation seems unlikely, for the results suggest gonadotrophin insufficiency rather than interference with estrogen action.

[5] Unpublished data.

The inverse relationship between thyroid-gland weight and mammary-tumor genesis and mammary-gland development could be interpreted as an indication that an increase in thyrotrophin secretion brings about reduction in gonadotrophin secretion and, indirectly, in ovarian secretion. The size of the end organs—large thyroid gland, underdeveloped mammae or small thyroid gland, highly developed mammae, or presence of mammary tumors—lends support to such an interpretation.

Results obtained with gonadotrophin supplementation of a small group of thiouracil-treated animals would seem to indicate that there is a gonadotrophin deficiency.[5] These animals responded by showing increased mammary development, resulting in the eventual appearance of numerous hyperplastic nodules and, in some cases, of mammary tumors. Normal estrus also appeared, which indicated that the ovary was capable of functioning when adequate pituitary stimulation was available. These results, which were achieved during the administration of thiouracil, indicate that the initial underdevelopment of mammae, ovary, and uterus is due to deficient hormonal stimulation and cannot be attributed to possible toxic action of thiouracil.

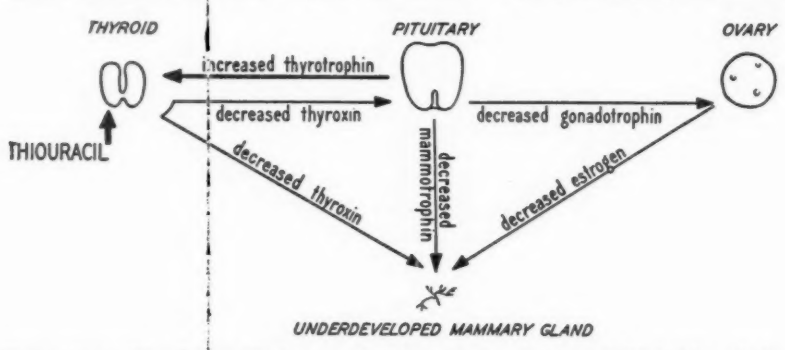
Mechanism of action.—The results of this experiment indicate that the metabolite antagonist, thiouracil, by blocking the action of an endocrine gland (thyroid) not directly responsible for mammary growth (*21*, *25*, *26*), initiates a sequence of changes manifested by decreased body growth, disruption of estrus, underdevelopment of the mammae, and, finally, reduced mammary-tumor genesis.

Since mammary tumors are considered to originate from the hyperplastic nodules (*27*, *28*) which generally arise from the acinar portions of the gland, any regimen that will tend to inhibit or delay development of the mammae and hyperplastic nodules will influence the genesis of mammary tumors. Presumably, of the factors thought necessary for mammary tumor formation only the hormonal factor was altered by the administration of thiouracil.

A deficiency of thyroxin and of some pituitary hormones might adversely affect protein anabolism to such an extent that some amino-acid deficiency would occur in spite of an adequate protein intake. If this situation did exist, these results might be explained by a hypophyseal insufficiency, produced either directly by insufficient amino acids to allow synthesis of tissue proteins for growth, or indirectly through failure in pituitary-hormone synthesis. Even though there was a decrease in size of some organs influenced by the trophic hormones, total failure in pituitary-hormone secretion apparently did not occur. This was evident by the greatly enlarged thyroid glands after prolonged thiouracil ingestion, indicating an abnormally large production of the thyrotrophic hormone. Although thyroxin deficiency probably contributed to the inhibition of mammary development, its influence may not have been as important as the lack of estrogenic or mammogenic hormones, for mammary development did take place in animals receiving thiouracil plus gonadotrophin.

Is the mechanism which produces inhibition of mammary tumor genesis in partial inanition or in specific nutritional deficiencies identical to that of thiouracil? The answer cannot be given with certainty at this time. The reduction in food intake of the thiouracil treated mice was thought to be insufficient to produce a deficiency of calories unless there was some interference with food utilization. The fact that more food was consumed by the thiouracil-treated mice per unit of body weight suggests poorer food utilization. Gye (*29*) has also stated the view that the effect of thiourea appeared to be more specific than that of simple underfeeding and that the long periods of diestrus and underdevelopment of the mammae resembled the behavior of mice after ovariectomy.

Another explanation is suggested involving production of a hormonal imbalance not attributable to poor nutrition. The pituitary gland in its



TEXT-FIGURE 10.—Schematic diagram to interpret the syndrome initiated by administration of thiouracil to C3H female mice.

attempt to secrete more and more thyrotrophin may not maintain its secretion of the other trophic hormones at a level sufficient to stimulate the adequate functioning of other endocrines. Although no quantitative determinations of specific hormonal levels were made, the following theoretical mechanism of action is suggested (text-fig. 10): Thiouracil blocks thyroxin formation. The pituitary response to the low thyroxin level is an increased elaboration of thyrotrophin. Although the thyroid gland increases in size, the thyroxin insufficiency continues. The pituitary gland maintains the production of large amounts of thyrotrophin, but only at the expense of other trophic hormones; lowered quantities of gonadotrophin and possibly mammotrophin are released. The lack of gonadotrophic stimulation interferes with ovarian function resulting in a reduced output of estrogen. Thus the hormones specifically concerned with mammary growth—estrogen and mammotrophin; and the general metabolic hormones—thyroxin and growth hormone, would be insufficient or lacking. Consequently the mammary glands fail to develop fully, hyperplastic nodule formation is reduced, and finally there is a suppression of mammary-tumor genesis.

SUMMARY

A metabolic antagonist, thiouracil, was used to interfere with the function of the thyroid gland, and the effect of this action on mammary growth and tumor genesis was investigated. The goitrogen was ingested by virgin C3H female mice from 9 weeks of age until death at a dietary level of 0.375 and 0.5 percent. The following results were obtained in the thiouracil-treated mice:

Growth of the treated mice ceased at an earlier age, and their average body weight was 3 to 9 grams lower than the control group. Less food was consumed by the treated mice but in proportion to body weight their average food intake exceeded that of the control group.

Estrus was inhibited and then completely suppressed for 8 to 15 weeks after initiation of treatment. Subsequently estrus reappeared at irregular and infrequent periods but the normal pattern was never re-established. Estrous activity was found to be a poor indicator of mammary-gland development.

Mammary development was arrested with the majority of animals showing no greater growth than was present at the start; only three animals achieved development comparable to that found in the adult untreated virgin female.

Hyperplastic nodules were reduced in number and size.

Mammary-tumor incidence was reduced to 19 percent of 52 mice, compared to 92 percent of 53 control mice.

The thyroid gland was extremely enlarged. Body weight, mammary-gland development, and mammary-tumor formation were inversely related to thyroid-gland weight.

A theoretical mechanism explaining the results is discussed, the essential features comprising: creation of a hormonal imbalance initiated by the inability of the thyroid gland to secrete thyroxin while under the influence of thiouracil; followed by a compensatory oversecretion of thyrotrophin by the pituitary gland and a concomitant undersecretion of other trophic hormones.

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PLATE 126

Whole mounts of mammary glands showing Stage A development; scattered buds and simple branching of ducts, very few side branches present.

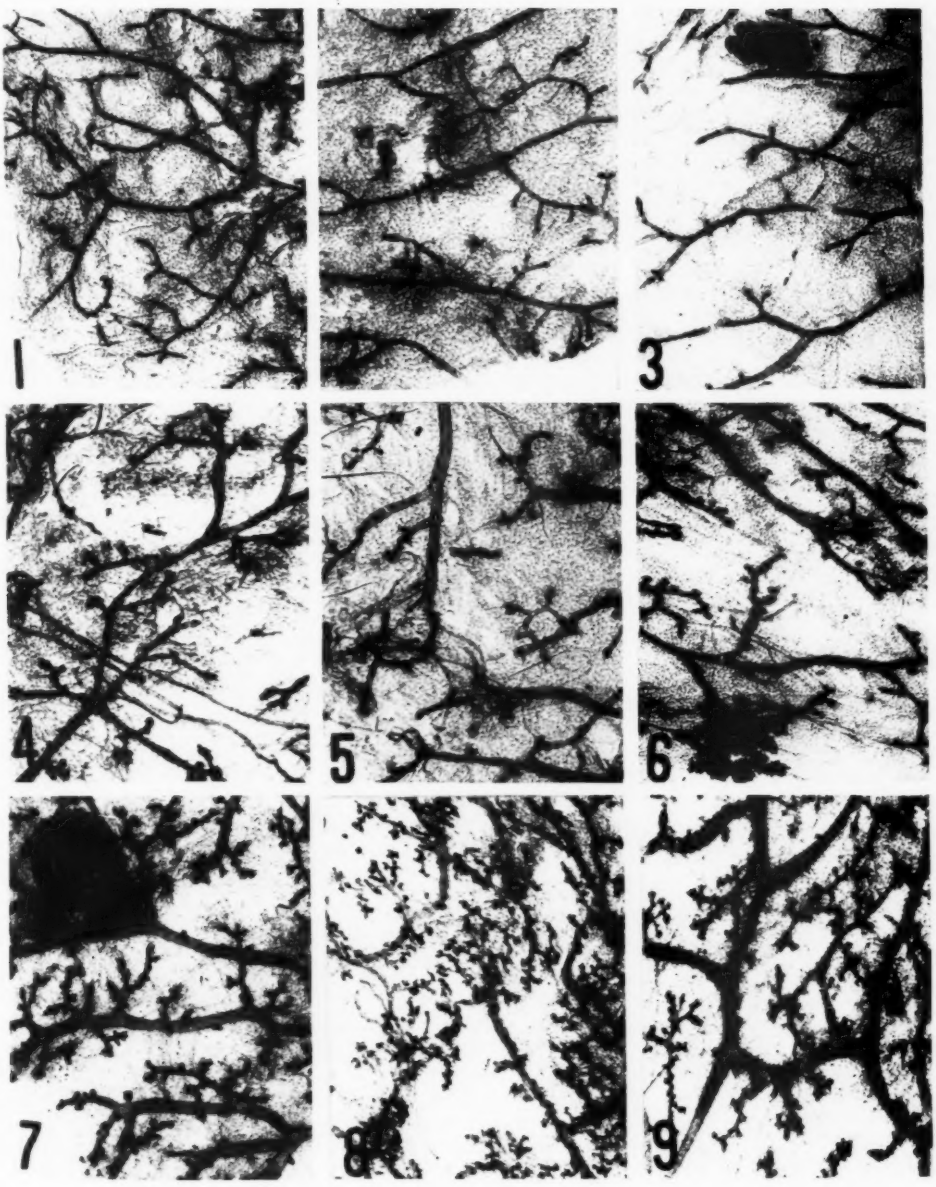
FIGURE 1.—Control animal, 56 days on stock diet, 119 days of age. × 15.
FIGURE 2.—Treated animal, 56 days on thiouracil diet, 119 days of age. × 15.
FIGURE 3.—Treated animal, 413 days on thiouracil diet, 476 days of age. × 15.

Whole mounts of mammary glands showing Stage B development: Increased number of buds, branching more complex, with some smaller caliber ducts present.

FIGURE 4.—Control animal, 76 days on stock diet, 139 days of age. × 15.
FIGURE 5.—Treated animal, 139 days on thiouracil diet, 202 days of age. × 15.
FIGURE 6.—Treated animal, 280 days on thiouracil diet, 343 days of age. × 15.

Whole mounts of mammary glands showing Stage C development: Very complex branching with profuse number of small caliber ducts.

FIGURE 7.—Control animal, 330 days on stock diet, 393 days of age. × 15.
FIGURE 8.—Treated animal, 76 days on thiouracil diet, 139 days of age. × 15.
FIGURE 9.—Treated animal, 370 days on thiouracil diet, 433 days of age. × 15.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EFFECT OF ULTRASONIC VIBRATION ON THE FORMED ELEMENTS OF BLOOD FROM NORMAL AND LEUKEMIC SUBJECTS [1] [2]

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INTRODUCTION

The resistance of erythrocytes and leukocytes to abnormal external conditions may be an important factor in their life span. The fragility of erythrocytes has been determined by exposing the cells to hypertonic and hypotonic solutions (*1*, *2*), hemolysins (*3*), agglutinins (*4*), varying osmotic tensions (*5*), heat (*6*), chemical (*7*, *8*), and mechanical agents (*9*). Little data are available concerning the fragility of leukocytes (*10*).

The development of apparatus which employ ultrasonic frequencies offers another tool for the examination of the effects of mechanical forces on cell structure. A study was made of the reaction of the formed elements of normal and leukemic bloods to an ultrasonic vibration of 635 kcps.

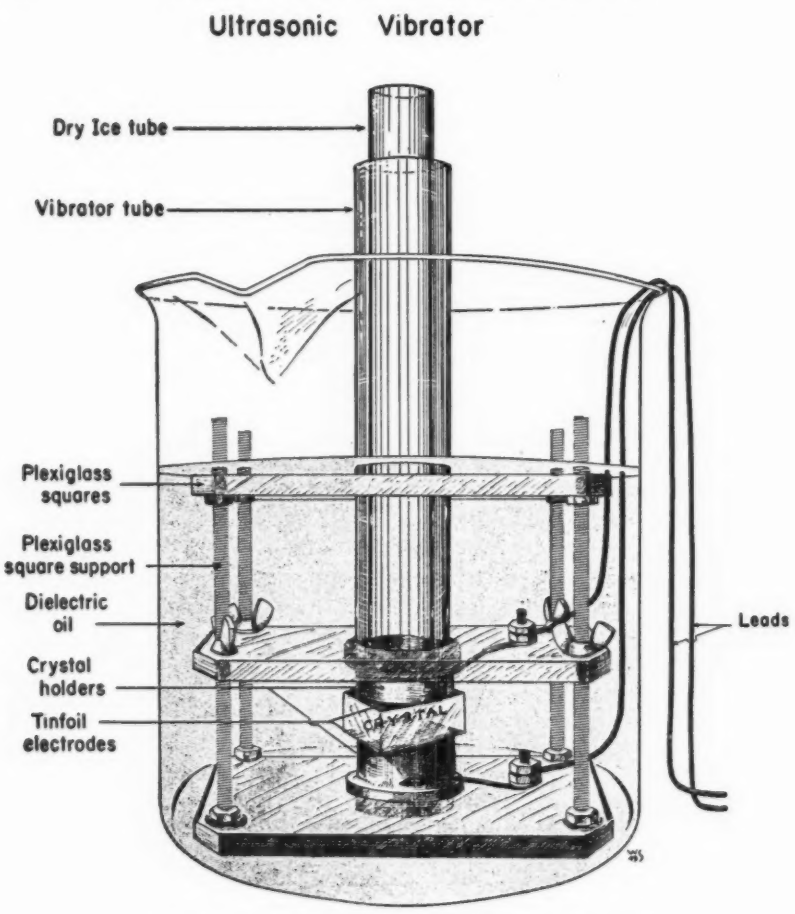
MATERIALS AND METHODS

The ultrasonic vibrator employed in this study consisted of 4 basic units: a Hartley electron-coupled oscillator; a power amplifier; a π-matching network; and a piezoelectric "X"-cut quartz crystal, 2.0 by 2.5 by 0.5 cm., with a natural resonant frequency of 635 kilocycles per second. Approximately 10,000 volts were applied to the crystal during the determinations, giving a power input to the oil bath of approximately 40 watts.

The crystal was set in a holder and immersed in an 18 by 15 cm. glass cylinder filled with a dielectric transformer oil (text-fig. 1). This holder consisted of 2 circular, hollow brass rings, approximately 2.5 cm. in diameter, each set in a 10 cm. plexiglass square. The crystal and tinfoil electrodes were held between the rings. The vibrator tube (a pyrex test tube 25 by 130 by 3 mm. thick), which contained the blood sample and the dry ice tube, rested in the top brass ring 5 mm. above the crystal, and was

[1] Received for publication August 17, 1949.

[2] The authors wish to thank Mr. Bruce Shumway for his valuable assistance in the construction of the ultrasonic vibrator.



TEXT-FIGURE 1.—Diagram of the ultrasonic vibrator.

held erect by a third plexiglass square. The same vibrator and dry ice tubes were used during all exposures.

The adjustment of the vibrator, the proximity of the vibrator tube to the crystal, and the heat generated were held constant at predetermined values during each exposure.

A 3-inch column of dry ice in a pyrex test tube (18 by 150 by 2 mm. thick) was found to maintain the blood at an average temperature of 30° C. during the vibration. Because of the small space between the dry ice tube and the vibrator tube, temperatures above 35° C. were found to produce varying degrees of hemolysis of the red blood cells, while temperatures below 20° C. were impractical due to frosting of the dry ice tube.

Tests were made on freshly drawn venous blood samples; 2 mg. of heparin was added to each 10 cc. of blood. The pH of the blood was

determined electrometrically before and after addition of the heparin, and after exposure to ultrasonic vibration, and was found to remain between 7.35 and 7.45.

A 2.5-cc. sample of the thoroughly mixed venous blood was exposed to the vibration immediately after the dry ice test tube had been placed in the blood contained in the vibrator tube. Counts and temperature readings during the exposure were taken at 2-minute intervals over a period of 10 minutes for the normal runs, and at intervals of 4 minutes over the period of the leukemic runs. Each sample was run in duplicate.

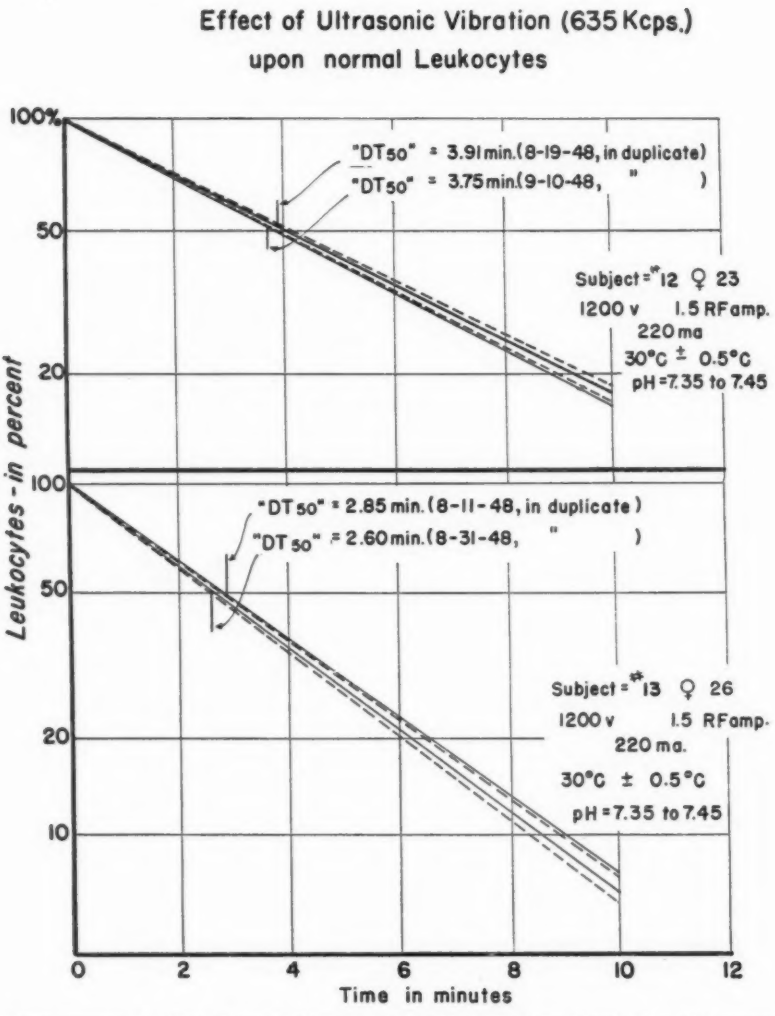
Samples for counts were taken in NBS Trenner Automatic pipettes; the same pipettes were used for each blood. Toison's solution was used as the diluting fluid for the red cell counts; Turk's solution for the white cell counts. The supravital technic was used for the differential counts. All pipettes were shaken in a mechanical rotator for 10 minutes before counting. Both sides of the Levy-Hauser NBS hemocytometer were counted, the average of the two determinations being used as the final figure.

RESULTS

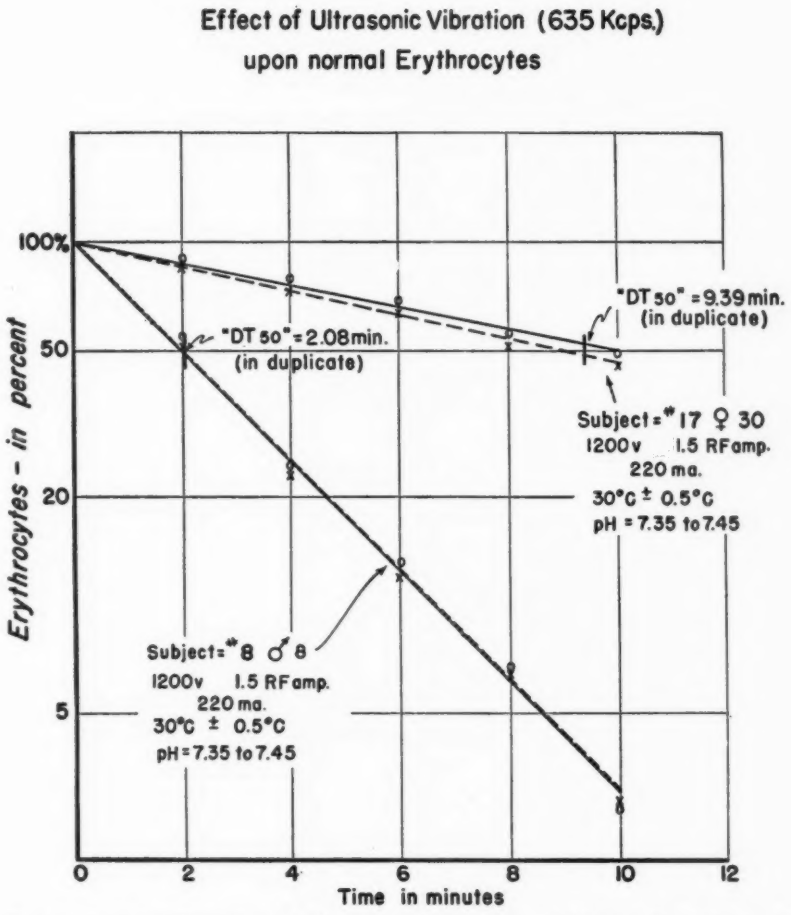
Sixty-four determinations were made on the rate of destruction of white blood cells in blood obtained from 30 normal individuals and 15 patients with myelogenous or lymphatic leukemia. The rate of destruction of red blood cells of the same blood was measured in 19 normal and 12 leukemic cases. In 18 normal bloods and 11 leukemic bloods, the studies were extended to observations of the differential destruction of lymphocytes and granulocytes.

In all instances, a plot of the percent of remaining cells against time gave a straight line on semi-logarithmic graph paper (text-figs. 2 and 3). On this basis, the time at which 50 percent of the cells were destroyed was determined from the curves, and this point designated as Destruction Time 50 percent, or DT_{50}. The terms "fragile" and "resistant" as used in this report refer to comparisons of the DT_{50}, and apply to the reaction of the cells to ultrasonic vibration under the specific test conditions described.

Normal Blood: The detailed data on the DT_{50} of erythrocytes and leukocytes of normal blood are given in table 1. The DT_{50} of polymorphonuclear neutrophiles and small lymphocytes are recorded in table 2; the initial absolute numbers of other leukocytes were too low for reliable quantitative determinations. The DT_{50} for erythrocytes on 19 consecutive duplicate runs ranged from 1.98 to 9.39 minutes. In 33 determinations of leukocytes, the DT_{50} varied from 1.69 to 6.75 minutes. Differential counts in 18 studies showed that the DT_{50} for the polymorphonuclear neutrophiles was from 1.83 to 7.30 minutes, whereas the DT_{50} for the small lymphocytes was from 1.80 to 4.20 minutes (table 3). Repeated determinations, 20 days to 8 months after the initial study, showed close reproducibility of the results (table 1).



TEXT-FIGURE 2.—The effect of ultrasonic vibration on white blood cells of 2 normal subjects. The samples were counted at 2-minute intervals. The DT_{50} was redetermined approximately 3 weeks later in each case.



TEXT-FIGURE 3.—The effect of ultrasonic vibration on red blood cells of 2 normal subjects. Samples were counted at 2-minute intervals.

TABLE 1.—*DT_{50} of erythrocytes and leukocytes exposed to ultrasonic vibration; normal subjects*

Subject	Sex	Age	Initial count		DT_{50}		Remarks
			RBC	WBC	RBC	WBC	
		Years	*Millions*	*Number*	*Minutes*	*Minutes*	
1	M	0. 13	3. 7	9, 000	4. 40	3. 12	
2	M	. 25	4. 87	11, 350	3. 19	2. 55	
3	F	. 5	4. 38	15, 200	5. 31	4. 30	
4	M	2	5. 04	7, 900	2. 80	2. 95	
5	F	4	4. 95	5, 600	2. 40	2. 30	
6	F	6	4. 3	8, 000	2. 29	1. 69	
7	F	7	4. 1	8, 200	3. 29	3. 70	
8	M	8	4. 9	7, 500	2. 08	2. 05	
9	M	9	5. 04	7, 450	1. 98	2. 42	
10	F	12	4. 2	6, 800	2. 90	2. 64	
11	M	21	------	6, 200	------	6. 75	
12	F	23	------	5, 750	------	3. 91	LMP—14 da.[1]
			4. 74	5, 800	5. 80	3. 75	Repeat 22 da. LMP—2 da.
13	F	26	------	6, 000	------	2. 85	LMP—6 da.
				6, 700	------	2. 60	Repeat 20 da. LMP—26 da.
				7, 500	------	2. 84	Repeat 8 mo. LMP—22 da.
14	F	27	------	5, 650	------	4. 08	LMP—3 da.
15	F	28	------	6, 700	------	3. 68	LMP—20 da.
16	M	30	------	7, 000	------	3. 00	
17	F	30	4. 01	7, 000	9. 39	5. 25	LMP—19 da.
18	M	33	------	6, 650	------	6. 60	
19	M	33	5. 4	6, 450	7. 00	4. 68	
20	M	35	------	6, 900	------	3. 50	
21	F	59	4. 24	8, 350	4. 90	3. 81	
22	M	60	------	5, 650	------	3. 00	
23	F	60	3. 55	5, 250	4. 39	4. 70	
24	M	62	------	6, 100	------	4. 23	
25	F	62	3. 05	7, 550	2. 32	1. 79	
26	M	65	4. 61	10, 050	4. 35	3. 27	
27	F	65	4. 48	6, 500	3. 71	2. 75	
28	F	69	3. 92	7, 050	3. 35	2. 50	
29	M	70	------	8, 900	------	2. 53	
30	M	73	------	6, 600	------	2. 30	

[1] LMP—days after last menstrual period.

The statistical analysis [3] of the destruction of the polymorphonuclear neutrophiles indicates that those of the adult group (21 to 35 years) are more resistant to ultrasonic vibration than those of the other two age groups. The difference between the youngest and oldest groups was not significant. The small lymphocytes of the adult group are not more resistant, however. No significance was demonstrated for these three age groups.

A comparison of the polymorphonuclear neutrophiles with the small lymphocytes shows that only the polymorphonuclear neutrophiles of the adult group (21 to 35 years) are more resistant to ultrasonic vibration. No significance was demonstrated when the polymorphonuclear neutrophiles of the youngest and oldest group were compared with the small lymphocytes.

[3] Statistical significances were determined by Fisher's "t" method. The level in cases where the difference was significant was 1 percent or less; in cases where the difference was not significant, 25 percent or more.

TABLE 2.—*DT_{50} of polymorphonuclear neutrophiles and small lymphocytes exposed to ultrasonic vibration; normal subjects*

Subject	Sex	Age	Initial WBC count	DT_{50}		Remarks
				PMN	Lym	
		Years	*Number*	*Minutes*	*Minutes*	
2	M	0. 25	11, 350	3. 35	1. 80	
4	M	2	7, 900	3. 13	2. 90	
5	F	4	5, 600	2. 28	2. 55	
6	F	6	8, 000	1. 83	1. 90	
7	F	7	8, 200	4. 00	3. 84	
9	M	9	7, 450	2. 35	1. 90	
10	F	12	6, 800	2. 70	3. 09	
12	F	23	5, 750	4. 54	3. 12	LMP—14 da.[1]
13	F	26	6, 700	2. 82	2. 00	LMP—26 da.
15	F	28	6, 700	4. 39	3. 23	LMP—20 da.
16	M	30	7, 000	3. 63	2. 31	
17	F	30	7, 000	7. 30	4. 20	LMP—19 da.
19	M	33	6, 450	6. 07	3. 15	
21	F	59	8, 350	4. 59	3. 61	
22	M	60	5, 650	3. 84	2. 70	
26	M	65	10, 050	3. 30	3. 60	
27	F	65	6, 500	3. 24	2. 90	
28	F	69	7, 050	2. 80	2. 90	

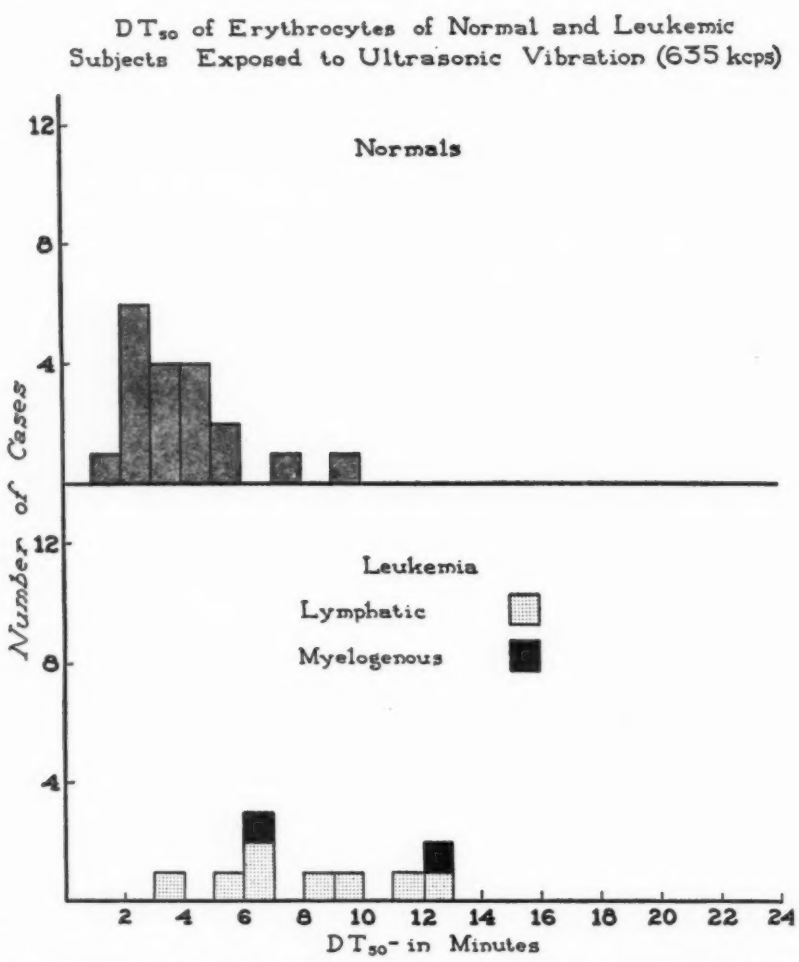
[1] LMP—days after last menstrual period.

TABLE 3.—*Statistical evaluation of the DT_{50} of erythrocytes and leukocytes of normal and leukemic subjects*

Blood cell type	Age group	Observations	Range	Mean	Standard deviation
Normal	*Years*	*Number*	*Minutes*		
Leukocytes	0. 13–73	33	1. 69–6. 75	3. 41	1. 22
Polymorphonuclear Neutrophiles	0. 25–69	18	1. 83–7. 30	3. 68	1. 36
Small Lymphocytes	0. 25–69	18	1. 80–4. 20	2. 87	. 70
Erythrocytes	0. 13–69	19	1. 98–9. 39	3. 95	1. 90
Lymphatic leukemia					
Leukocytes	2–68	13	6. 60–22. 30	12. 65	2. 37
Polymorphonuclear Neutrophiles	2–68	4	2. 30– 6. 75	4. 10	1. 76
Small Lymphocytes	2–68	8	6. 40–31. 00	14. 43	9. 00
Erythrocytes	2–66	8	3. 64–13. 00	-------	------
Myelogenous leukemia					
Leukocytes	31–62	6	8. 20–23. 00	13. 60	2. 11
Polymorphonuclear Neutrophiles	31–61	2	7. 00–13. 80	-------	------
Small Lymphocytes	61	1	14. 40	-------	------
Erythrocytes	31–62	2	6. 80–12. 70	-------	------

There is no correlation of the sex of the subject with the fragility of the erythrocytes or leukocytes. No relation was apparent between the fragility of blood cells and the time from the last menstrual period.

No correlation was demonstrated between the DT_{50} of the erythrocytes or leukocytes and the initial number of cells in the blood sample. The



TEXT-FIGURE 4.—Relation and variation of DT_{50} of erythrocytes of normal, lymphatic, and myelogenous leukemia cases.

blood of animals containing more resistant leukocytes does not necessarily have more resistant erythrocytes, nor does the blood with more resistant erythrocytes have more resistant leukocytes.

Lymphatic Leukemia: Of the 11 cases of lymphatic leukemia, 2 cases were 4 months after treatment with methyl-bis (beta-chloroethyl)amine hydrochloride (HN2), and 1 case was during treatment with aminopterin. The remaining 8 cases were untreated.

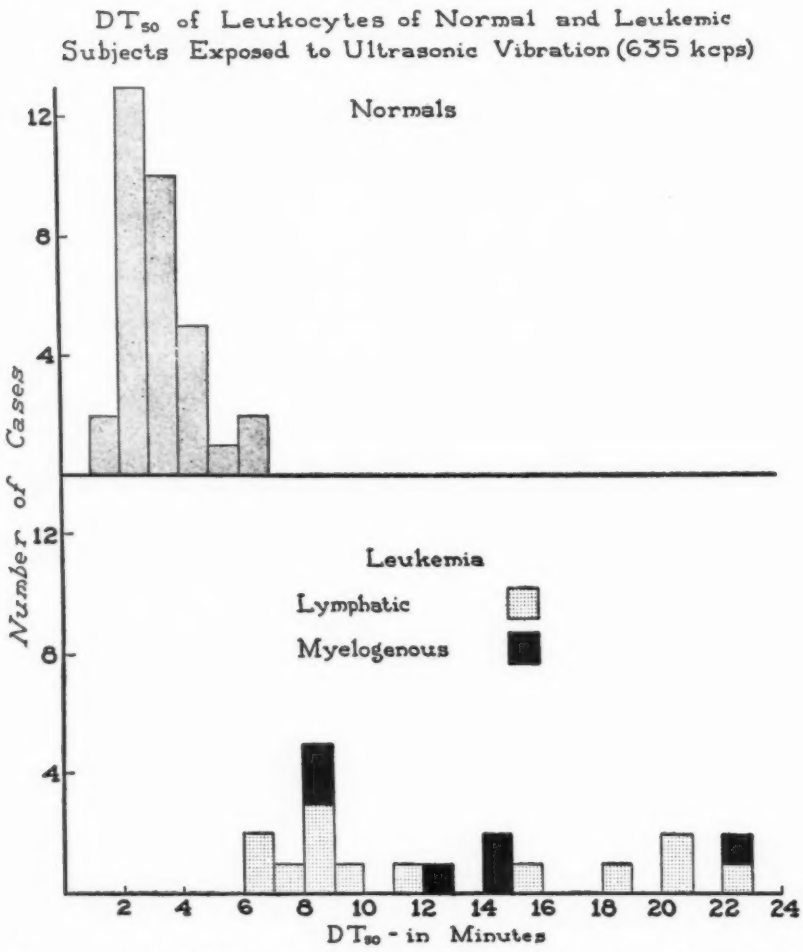
A comparison of the DT_{50} of the erythrocytes and leukocytes according to the age and sex of the subjects and the initial blood count again showed no correlation.

The DT_{50} of the erythrocytes in 8 consecutive duplicate runs ranged from 3.64 to 13.00 minutes (table 4). The DT_{50} of the erythrocytes was normal except in 2 cases of chronic lymphatic leukemia (1 untreated and the other 4 months after x-ray), and in 1 untreated case of acute lymphatic leukemia. The DT_{50} of the 2 chronic cases was 11.50 and 13.00 minutes respectively, while the DT_{50} of the acute case was 9.50 minutes (text-fig. 4).

TABLE 4.—*DT_{50} of erythrocytes and leukocytes exposed to ultrasonic vibration; leukemic subjects*

Subject	Sex	Age	Initial count		DT_{50}		Remarks
			RBC	WBC	RBC	WBC	
		Years	*Millions*	*Number*	*Minutes*	*Minutes*	
LYMPHATIC LEUKEMIA							
31	F	60	2. 33	40, 000	6. 45	18. 90	Chronic—untreated.
				13, 150	.....	2. 99	11 da post HN2.
32	M	66	.....	295, 000	.....	20. 60	Chronic—untreated.
				287, 000	.....	20. 00	Repeat—7 da during treatment with x-ray.
				44, 000	.....	6. 09	
33	M	68	.....	308, 000	.....	8. 35	Chronic—untreated.
34	F	66	3. 90	109, 500	11. 50	22. 30	Chronic—untreated.
35	M	35	3. 60	120, 000	3. 64	9. 80	Chronic—4 mo. post HN2.
				45, 000	.....	10. 10	16 da post p^{32}.
				14, 600	.....	8. 70	21 da post p^{32}.
36	M	57	3. 27	14, 250	13. 00	11. 50	Chronic—4 mo. post x-ray.
37	M	2	2. 19	30, 000	8. 40	6. 65	Acute—untreated.
38	M	30	3. 80	14, 450	5. 80	15. 25	Acute—untreated.
39	F	35	3. 37	38, 200	6. 90	6. 60	Acute—untreated.
40	F	35	2. 91	176, 000	9. 50	8. 10	Acute—untreated.
				181, 750	.....	7. 90	Repeat—2 da.
41	F	8	.....	92, 000	.....	8. 50	Chronic—during treatment with aminopterin.
MYELOGENOUS LEUKEMIA							
42	F	61	.....	195, 000	.....	14. 10	Chronic—untreated.
				183, 000	.....	14. 80	Repeat—1 da.
				252, 000	.....	11. 40	5 da post p^{32}.
				240, 000	.....	11. 75	12 da post p^{32}.
				164, 500	.....	7. 25	20 da post p^{32}.
				80, 000	.....	6. 40	30 da post p^{32}.
				65, 500	.....	6. 70	35 da post p^{32}.
43	F	58	.....	76, 000	.....	8. 20	Chronic—4 mo. post x-ray.
				96, 000	.....	8. 50	Repeat—6 da.
			2. 89	37, 000	12. 80	4. 60	During aminopterin.
			2. 97	120, 750	12. 20	5. 60	23 da post aminopterin.
44	F	62	3. 41	212, 000	6. 80	23. 00	Acute—untreated.
45	F	31	2. 43	13, 550	12. 70	13. 00	Acute—untreated.
				228, 500	.....	10. 00	4 mo. post aminopterin.

In 13 determinations of leukocytes, the DT_{50} varied from 6.60 to 22.30 minutes. Repeated determinations 2 and 7 days after the initial study showed close reproducibility of the results (table 4). A comparison of the DT_{50} of the leukocytes of the 11 cases of lymphatic leukemia with the DT_{50} of the normal leukocytes showed a statistically significant increased time of survival of the leukocytes of lymphatic leukemia (text-figure 5, table 3). Three cases of chronic lymphatic leukemia were followed after treatment (table 4). Two untreated cases showed reduction of the DT_{50}



TEXT-FIGURE 5.—Relation and variation of DT_{50} of leukocytes of normal, lymphatic, and myelogenous leukemia cases.

of the leukocytes from 18.90 to 2.99 minutes in 11 days after treatment with HN2, and from 20.60 to 6.09 minutes in 32 days after the beginning of x-ray treatment. The remaining case showed a reduction of the DT_{50} from 9.80 to 8.70 minutes 21 days after treatment with radioactive phosphorus (P^{32}). This reduction of the DT_{50} does not seem to depend on a reduction in count, since the drop in count is not proportional to the drop in resistance.

Differential studies showed that the DT_{50} of the polymorphonuclear neutrophiles varied from 2.30 to 6.75 minutes; the DT_{50} of the small lymphocytes was from 6.40 to 31.00 minutes (table 5). The DT_{50} of the

polymorphonuclear neutrophiles of 4 cases of lymphatic leukemia was not significantly longer than those of normal polymorphonuclear neutrophiles (table 3). In no case was the DT_{50} of the polymorphonuclear neutrophiles above the normal range. The small lymphocytes of 8 cases of lymphatic leukemia, however, showed a statistically significant increased DT_{50} when compared to the DT_{50} of normal small lymphocytes (table 3). In every case the DT_{50} of the leukemic small lymphocytes was above the normal range.

Myelogenous Leukemia: Three of the four cases of myelogenous leukemia were untreated; the remaining case was 4 months after treatment with x-ray.

A comparison of the DT_{50} of the erythrocytes and leukocytes according to the age of the subjects and the initial blood count again showed no correlation.

TABLE 5.—*DT_{50} of granulocytes and lymphocytes exposed to ultrasonic vibration; leukemic subjects*

Subject	Sex	Age	Initial WBC Count	DT_{50}					
				PMN	Band	Meta	Myel C	Myel A	Blast
LYMPHATIC LEUKEMIA									
		Years	*Number*	*Minutes*	*Minutes*	*Minutes*	*Minutes*	*Minutes*	*Minutes*
31	F	60	40, 000	------	------	------	------	------	------
32	M	66	287, 000	------	------	------	------	------	------
33	M	68	308, 000	2. 30	------	------	------	------	------
34	F	66	109, 500	6. 75	------	------	------	------	------
36	M	57	14, 250	------	------	------	------	------	------
37	M	2	30, 000	2. 75	------	------	------	------	------
39	F	35	38, 200	4. 60	------	------	------	------	------
40	F	35	181, 750	------	------	------	------	------	------
MYELOGENOUS LEUKEMIA									
42	F	61	195, 000	13. 80	12. 50	17. 90	12. 20	------	------
44	F	62	212, 000	------	------	18. 70	20. 00	13. 00	16. 00
45	F	31	13, 550	7. 00	4. 10	0. 90	2. 15	------	------

Subject	Sex	Age	Initia lWBC Count	DT_{50}				
				PMB	PME	Lym-s	Lym-int	Lym-large
LYMPHATIC LEUKEMIA								
		Years	*Number*	*Minutes*	*Minutes*	*Minutes*	*Minutes*	*Minutes*
31	F	60	40, 000	------	------	31. 00	5. 60	------
32	M	66	287, 000	------	------	21. 50	------	------
33	M	68	308, 000	------	------	8. 75	------	------
34	F	66	109, 500	------	------	20. 00	25. 25	6. 50
36	M	57	14, 250	------	------	14. 10	5. 40	------
37	M	2	30, 000	------	------	7. 10	2. 50	------
39	F	35	38, 200	------	------	6. 40	4. 00	------
40	F	35	181, 750	------	------	6. 60	9. 25	------
MYELOGENOUS LEUKEMIA								
42	F	61	195, 000	17. 10	16. 20	14. 40	------	------
44	F	62	212, 000	------	------	------	------	------
45	F	31	13, 550	------	------	------	------	------

The DT_{50} for erythrocytes in 2 consecutive duplicate runs was 6.80 and 12.70 minutes (table 4). The DT_{50} of the erythrocytes was within the range of the normals in 1 acute untreated case. In the other case the DT_{50} was above normal, being 12.70 minutes (text-figure 4).

On 6 determinations of leukocytes, the DT_{50} varied from 8.20 to 23.00 minutes. Repeated determinations, 1 and 6 days after the initial study, showed close reproducibility of the results (table 4). The DT_{50} of the leukocytes of 4 cases of myelogenous leukemia was significantly longer than leukocytes of normal subjects (text-fig. 5, table 3). Three cases of myelogenous leukemia were followed after treatment (table 4). An acute untreated case with an original DT_{50} of 13.00 minutes dropped to 10.00 minutes 4 months after treatment with aminopterin. A chronic case with a DT_{50} of 8.20 minutes dropped to 4.60 minutes during treatment with aminopterin and was 5.60 minutes 23 days after the course of treatment. The third, a chronic untreated case, began with a DT_{50} of 14.10 minutes, dropped to 11.40 minutes 5 days after P^{32}, 11.75 minutes 12 days after, 7.25 minutes 20 days after, 6.40 minutes 30 days after, and was still 6.70 minutes 35 days after the treatment. This lessened resistance does not seem to depend on the count, since the DT_{50} in all 3 cases was lowered while the white count increased above the original count.

Differential studies showed that the DT_{50} of the polymorphonuclear neutrophiles ranged from 7.00 to 13.80 minutes (table 5). The DT_{50} of the polymorphonuclear neutrophiles of 2 of the cases of myelogenous leukemia was significantly increased over that of normal polymorphonuclear neutrophiles (table 3). Although no statistical evaluation could be made of the younger cells of the myeloid series, 2 cases showed DT_{50} of the younger cells consistently above the normal values for the adult cells. The DT_{50} of the band forms ranged from 4.10 to 12.50 minutes, the polymorphonuclear basophiles from 13.00 to 17.10 minutes, and the polymorphonuclear eosinophiles from 16.00 to 16.20 minutes. The metamyelocytes ranged from 0.90 to 18.70 minutes, and the myelocyte "C" forms from 2.15 to 20.00 minutes. In the one case where the initial small lymphocyte count was adequate, the DT_{50} was 14.40 minutes.

DISCUSSION

The physical and biological effects produced by ultrasonic vibration have been studied by many investigators (*11*, *12*, *13*, *14*). The lethal effects caused by ultrasonic vibration are generally attributed to changes produced within the cell by a mechanical force (*15*). This force involves cavitation, or the expulsion of dissolved gas from the medium (*16* to *20*). Coagulation of the protoplasm (*21*), and violent agitation produced within the cell by increased pressure changes, may also be contributory factors (*22*, *23*, *24*).

The data presented substantiate a previous report by Chambers and Gaines of the logarithmic rate of destruction of erythrocytes by ultrasonic vibration (*24*), and further demonstrate a similar logarithmic rate of reduction of the leukocytes. The reproducibility of the DT_{50} for erythro-

cytes and leukocytes from the normal and leukemic bloods was confirmed in all cases (tables 1 and 4).

Consistent changes in the appearance of the normal white blood cells occurred during the exposures (fig. 1*A*, *B*, *C*). After 2 to 4 minutes of vibration the lymphocytes were distorted, and some appeared smaller in size. The polymorphonuclear neutrophiles also were distorted but did not appear reduced in size at this time, although a few free granular fragments were present. After approximately 6 minutes the lymphocytes, although still whole, showed increased distortion. The myeloid cells were smaller and possessed few granules (fig. 1*B*). The few eosinophiles seen were misshapen, and free eosinophile granules were easily recognized. Numerous free nuclei with attached pieces of cytoplasm were observed. As the destruction progressed, sheets of broken cells appeared, and clumps of unidentifiable nuclei and granules were seen (fig. 1*C*).

The red blood cells did not change so markedly. Some crenation appeared at 2 to 4 minutes, but the majority of cells retained their normal appearance. The cells were noticeably reduced in number at 6 minutes, and the liberated hemoglobin gradually colored the field. The remaining red cells appeared remarkably normal, although an occasional red cell ghost could be seen. Hemolysis could be detected grossly after 4 to 10 minutes, depending on the individual red blood cell resistance. The viscosity of the blood appeared normal during the exposure.

The changes in the appearance of the leukemic blood cells which occurred during the exposures to ultrasonic vibration were similar to the changes observed in the normal cells. The distortion and condensation of the small lymphocytes occurred in both lymphatic and myelogenous leukemia, although the small lymphocytes from cases of lymphatic leukemia seemed to be affected more slowly and to a lesser degree. The granulocytes which were observed in lymphatic leukemias were distorted but did not appear reduced in size, and the granular fragments, free nuclei, and matting of cell fragments observed in the normal smears were also present in leukemic smears. The granulocytes in cases of myelogenous leukemia showed the same type of distortion and rupturing, although the changes appeared more slowly than in cases of lymphatic leukemia. Generally, the younger cells were more resistant to the vibration, especially where the early cell was the predominant type.

The changes occurring in the red blood cells from cases of leukemia were similar to those occurring in normal cells: slight crenation, no distortion, and liberation of hemoglobin as the cells were destroyed. Hemolysis of the erythrocytes could be detected grossly after 6 to 15 minutes, depending upon the resistance of the individual red blood cells.

A change in the viscosity of the blood during vibration was noted in 6 of the 11 cases of lymphatic leukemia (31, 35, 38, 39, 40, 41—table 4), and in all 4 cases of myelogenous leukemia. The change ranged from a slight thickening after 3 minutes of vibration (31—untreated) to an almost gelatinous consistency after 10 minutes of vibration (45—untreated). In

2 of the cases followed after treatment (35 and 42) the change of viscosity disappeared at 16 and 20 days after treatment, respectively.

This study demonstrates a markedly increased resistance of the leukocytes of lymphatic and myelogenous leukemia to one type of physical force, namely, ultrasonic vibration of 635 kcps. The data suggest that only the major cell type involved in the leukemia has this property, *e. g.*, the lymphocytes in lymphatic leukemia, while the erythrocytes and granulocytes are not affected. Whether this implies an increased survival of these cells in the circulating blood is merely speculative. Indeed, the results may apply only to the specific experimental conditions that were utilized. Szilárd (*25, 26*), for example, exposed normal and leukemic cells to normal and leukemic plasma and serum and states that leukemic leukocytes have a lower resistance which leads to their rapid destruction.

The increased resistance of leukemic leukocytes demonstrated by the present study is not in keeping with the common observation of large numbers of smudge cells on stained smears of leukemic blood. It is to be noted, however, that there are more smudge cells seen with the more acute types of leukemia in contrast to the chronic variety. There is a similar indication of longer DT_{50} of cells of the chronic leukemic patients than with those of the acute cases.

These studies do not preclude factors external to the cells, although this is unlikely in view of the absence of correlation of change in DT_{50} between erythrocytes and leukocytes and the absence of correlation of change in the viscosity of the blood.

SUMMARY

The effect of ultrasonic vibration of 635 kcps on formed elements of heparinized human blood was studied *in vitro* on blood obtained from 30 normal subjects and 15 patients with leukemia.

The erythrocytes and leukocytes of blood from normal and leukemic subjects were destroyed at a logarithmic rate.

The times at which 50 percent of the cells were destroyed (DT_{50}) were as follows:

1. Erythrocytes from 19 normal subjects, 1.98 to 9.39 minutes; leukocytes from 30 normal subjects, 1.69 to 6.75 minutes.

2. Erythrocytes from 8 cases of lymphatic leukemia, 3.64 to 13.00 minutes; leukocytes from 11 cases of lymphatic leukemia, 6.60 to 22.30 minutes.

3. Erythrocytes from 3 cases of myelogenous leukemia, 6.80 to 12.80 minutes; leukocytes from 4 cases of myelogenous leukemia, 8.20 to 23.00 minutes.

The DT_{50} of the leukocytes of both lymphatic and myelogenous leukemias is significantly greater than the DT_{50} of leukocytes of normal subjects.

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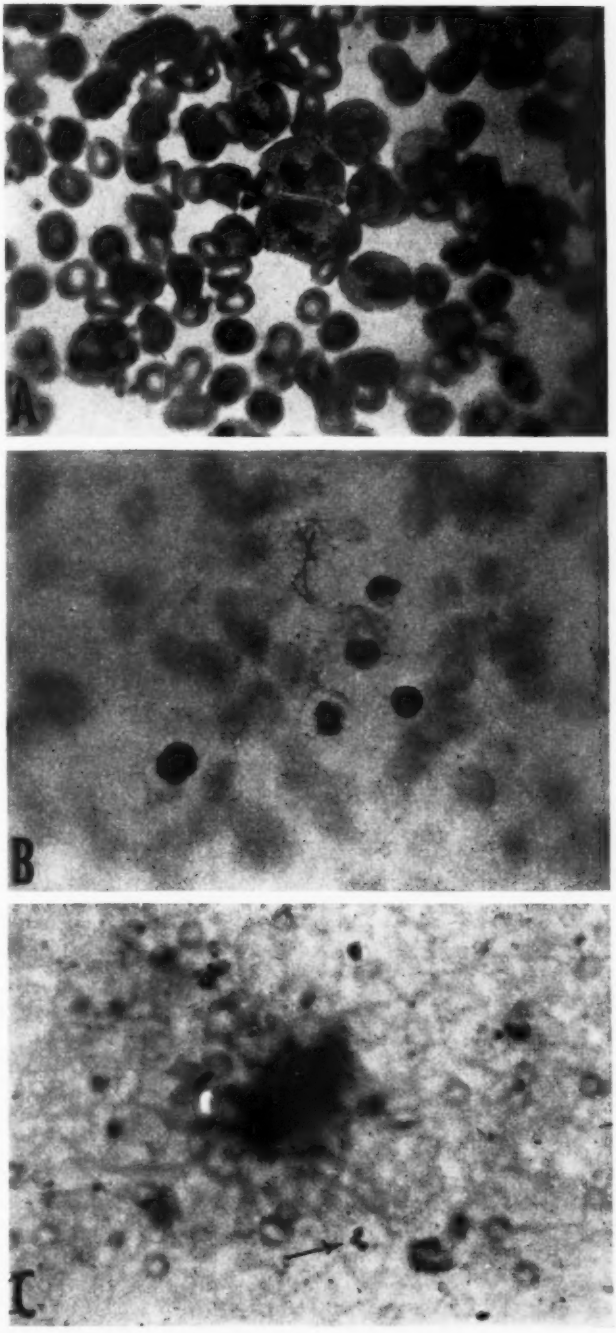
PLATE 127

FIGURE 1.—The effect of ultrasonic vibration on the peripheral blood cells (Case 21). All photographs taken under oil at ×-630 magnification. *A.* Mature polymorphonuclears and red blood cells before vibration. *B.* After 6 minutes of 635 kcps vibration. Note decrease in size of polymorphonuclears with lack of granulation, free cytoplasmic debris and decrease in number of erythrocytes. Lymphocytes do not decrease in size as rapidly as the polymorphonuclears. *C.* After 10 minutess nuclear and cytoplasmic debris with large clump of nuclear material in center. The intact erythrocytes are markedly few in a field of matrix-like background. Note 3-lobed nuclear fragment approximately 40 microns from the central mass (arrow) with clear surrounding area.


A
B
C

MAMMARY TUMOR-AGENT IN THE SPERM OF HIGH-CANCER-STRAIN MALE MICE [1]

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Hybridization experiments with mice have revealed that "whenever low-mammary-tumor-strain females have been mated to high-mammary-tumor-strain males, the incidence of mammary tumors among the hybrid females has been consistently raised above that of the low-mammary-tumor parent strain" [Heston (1)]. It is still an open question to what degree the mammary tumor-agent is involved in the production of these tumors and, if so, how it gained access to the hybrids. Andervont and Dunn (*2*, *3*) have adequately discussed this problem. Thus far, mothers' milk is the only known route for natural transmission of the mammary tumor-agent. However, the mammary tumor-agent is widely distributed throughout the body of male and female mice of high-cancer strains (*4–6*). Hence, it is conceivable that the mammary tumor-agent is transmitted from high-cancer-strain males to low-cancer-strain females and is responsible for the higher tumor rates in the hybrids. One possibility is that the agent is transmitted by the sperm [3] in copulation. Therefore, experiments were performed to test the sperm of high-cancer-strain males for the presence of the mammary tumor-agent.

MATERIALS AND METHODS

All mice were raised in this laboratory and fed commercial pellets and tap water *ad libitum*. The following strains of mice and hybrids were used:

d=dba strain (Dilute brown, Murray-Little) high-mammary-tumor strain;

B=C57 black (Little) low-mammary-tumor strain;

Bd, hybrids from mating B females to d males;

Odz, hybrids from mating 020 Leeuwenhoek-huis females to dz males. Strain 020 Leeuwenhoek-huis is a low-mammary-tumor

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[2] Under the direction of Dr. R. Korteweg.

[3] Throughout this paper the term "sperm" means the spermatozoa containing secretions of the testis and the epididymis. "Spermatozoon" means the male germ cell.

strain (7). Dz males are derived from an agent-free line which was procured by taking the young from the uterus of a pregnant d mouse and foster nursing them on a female of the B strain. The young were further bred by constant brother-sister mating. Animals of this line are designated by the letter z=dz.

TABLE 1.—*Summary of results. Injection of sperm from high-cancer-strain males into sensitive hybrids*

	Total number	With mammary tumors			Nontumor-bearing	
		Number	Percent	Average age in days	Number	Average age at death
A. UNTREATED CONTROLS						
♀ d-virgins	87	51	59	537	36	540
♀ d-breeders	122	71	59	439	51	559
♀ B-virgins	26	0	0	------	26	672
♀ B-breeders	14	0	0	------	14	627
♀ Bd forc.bred	29	1	3	565	28	706
♀ Odz forc.bred	35	0	0	------	* 35	593
B. TREATMENT WITH SPERM						
1. with sperm from ♂ d:						
♀ Bd	8	5	64	531	3	617
♀ Odz	16	13	81	392	3	559
2. with sperm from ♂ B:						
♀ Odz	7	0	0	------	* 7	519
C. TREATMENT WITH MILK-FACTOR						
♀ Bd } foster nursed on ♀ d	39	21	54	552	18	611
♀ Odz } foster nursed on ♀ d	16	16	100	423	------	------
D. TREATMENT WITH BLOOD						
1. with blood from ♂ d:						
♀ Odz	9	7	77	432	2	519
2. with blood from ♂ B:						
♀ Odz	9	0	0	------	* 9	495

* These mice are still living.

Donors of the sperm were d males 6–12 months old. The sperm was taken from the cauda epididymidis. The cauda was carefully dissected out on both sides in such a way as to avoid any soiling by blood. With fine scissors the cauda was then slit and the parts were immersed in 0.1 ml. of physiological salt solution, particles of epididymal tissue being prevented from getting into the solution. The presence of spermatozoa was checked microscopically. The sperm-containing solution was then injected intraperitoneally into Bd or Odz hybrids. In each animal the injection was performed three times: on the first to the second day of life, on the seventh to the tenth day, and on the eighteenth to the twentieth day, each animal thus receiving the sperm of three different d males. Controls were:

(1) ♀ Bd and ♀ Odz hybrids untreated;
(2) ♀ Odz hybrids treated in the same way with sperm from males of the B noncancer strain;
(3) ♀ Bd and ♀ Odz hybrids foster nursed on females of the d strain immediately after birth;
(4) ♀ Odz hybrids treated with blood from males of the d and the B strain. The blood was obtained by cardiac puncture and, without further treatment, 0.05–0.1 cc. of it was immediately injected intraperitoneally into the hybrids on the first to the second, on the seventh to the tenth, and on the eighteenth to the twentieth day.

After weaning, the test animals and the controls were mated with their untreated brothers and allowed to have three litters which were killed soon after birth (force-breeding). Observations were made at weekly intervals, tumors being noted as appearing on the date when first palpable. Histologic diagnoses were made in all cases. The animals listed in table 1 include only those that survived to tumor age as determined by the age of the first animal to develop a tumor.

DISCUSSION

It appears from this table 1 that the injection of sperm from the cauda epididymidis of high-cancer-strain males into sensitive hybrids gives rise to a high percentage of mammary tumors. The sperm of noncancer-strain males does not produce this effect.

In order to provide some information on the potency of the agent in the sperm, the same hybrids were foster-nursed throughout the entire lactation period on females of the cancer strain. The milk factor administered in this way produces, in these sensitive hybrids, a high percentage of mammary tumors in later life.

In another experiment, blood from both cancer-strain and noncancer-strain males was injected into the same hybrids. It is well known that the blood of animals of the cancer strains also contains the agent.

Comparison of the tumor percentages in animals treated with sperm and with the milk factor and with blood reveals a striking resemblance. It is safe to conclude, therefore, that the concentration of the agent in the sperm is certainly not minimal.

In the method used, great pains were taken to prevent blood or tissue particles of the cauda epididymidis from getting into the injection fluid, and in each individual case the presence of spermatozoa was checked microscopically. However, the possibility cannot be excluded that in the rinsing of the cauda minimal amounts of secretion may also have been rinsed out. It remains an open question, therefore, whether the secretions from the cauda or the spermatozoa themselves are the vectors of this agent. This problem requires further study.

While this investigation was in progress, Andervont and Dunn (*2*) reported that the seminal vesicles and contents of high-cancer-strain males also contain the agent. Seminal vesicles do not contain spermatozoa.

The contents of the seminal vesicles were not investigated separately. It is not yet known what role the secretion of the seminal vesicles plays in fertilization.

The finding of the mammary tumor-agent in the sperm makes it highly probable that transmission of the agent may take place in copulation. Should the transmission of the agent by the sperm be confirmed, the question then remains as to why only a few mammary tumors usually were observed in the offspring. Theoretically, quite a number of possibilities are conceivable. Among these, the fact that copulation does not occur until in later life is probably the most important, because administration of the agent in later life (after a lifetime of 6 weeks), even in susceptible animals, seldom or never gives rise to the development of mammary tumors (*8–9*). This resistance at a later age, however, may be overcome by repeated administration of the agent (*10*). Further studies for the elucidation of these problems are in progress.

SUMMARY

The sperm from the cauda epididymidis of high-cancer-strain male mice (dba Little) contains the mammary tumor-agent.

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PRODUCTION OF MALIGNANCY *IN VITRO*. IX. DESCRIPTION OF CELLS AT THE FLUID INTERFACE OF THE CULTURE [1] [2]

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INTRODUCTION

In an earlier article (*1*) experiments were described in which a parent strain of fibroblasts [3] from the subcutaneous fibrous and adipose connective tissue of a young adult male C3H mouse was originally planted *in vitro* October 18, 1940, and was grown in a medium of chicken plasma, horse serum, chick embryo extract, and saline solution. In this medium, cell strains taken from the parent strain were treated with 20-methylcholanthrene in a concentration of approximately 1 microgram of the carcinogen per milliliter of fluid medium for intervals ranging from 6 to 406 days.

Most of the work in this study was carried out on 6 cell strains. Five of these, designated as strains H, J, L, N, and O, were treated with the carcinogen for 6, 32, 111, 184, and 406 days respectively. A sixth strain designated as strain D was maintained untreated as a control. Morphologic alterations were observed in all treated strains. The nature of the change observed was such that the fibroblasts at the glass interface [4] of the culture shortened and became increasingly coherent to others laterally adjacent. In extreme stages of change seen in strains N and O, the cells of the growing edge of the culture often formed a closely coherent, sheetlike mass.

About 120 days after the last of the strains of treated cells had shown morphologic alterations, the cells of the untreated control cultures began to show a change of the same type, but much more limited in degree than in any of the treated strains. The cause of this change in the untreated controls was not indicated by the data available.

[1] Received for publication October 7, 1949.

[2] Technical assistance by Mary F. Brown, Thomas H. Stark, Nancy P. Straus, Rosslyn Wallace, Grady Carter, Leslie Hall, and Ellison Peppers.

[3] In this paper the original cell strain is designated the parent strain; each subordinate group of cultures which arose from it is designated as a strain. The term "fibroblast" is used to designate cells of the fibrous and adipose connective tissue having a typical fibroblastlike morphology as seen in tissue culture.

[4] In this article the terms "glass interface" and "fluid interface" of the culture are used to indicate the position of cells growing in the plasma clot adjacent to the glass surface of the culture flask and adjacent to the supernatant fluid culture medium respectively.

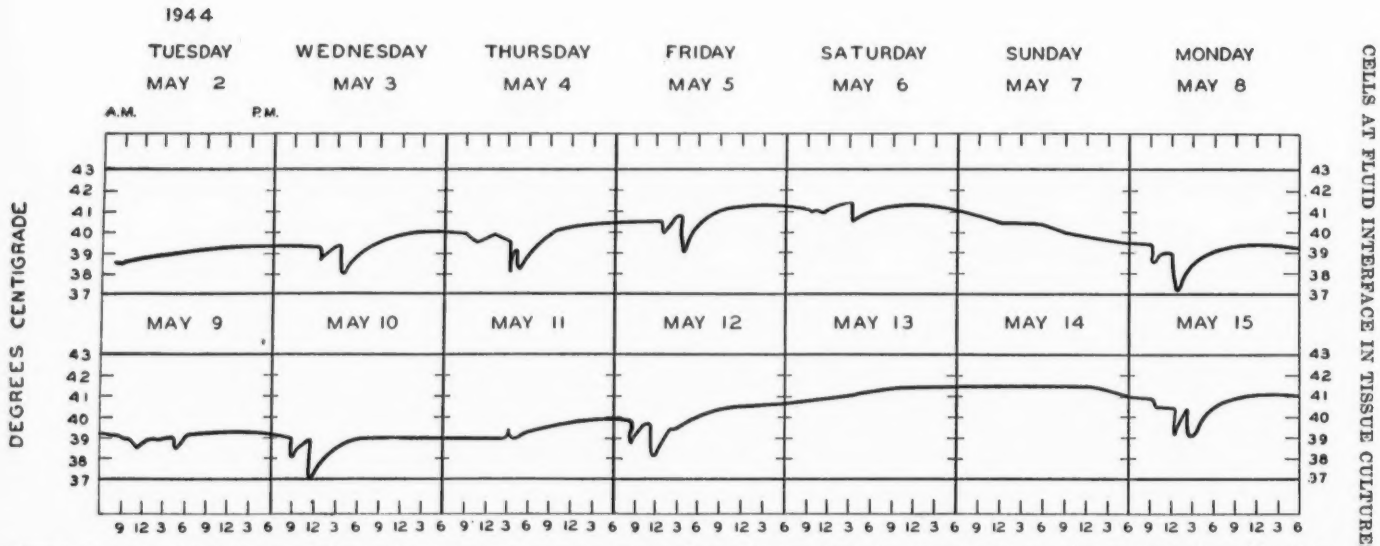
In the earlier study (*1*) little attention was given to the structure of the sheet of cells lying at the fluid interface of the culture. However, it was recognized that the appearance of the cell sheet at the fluid interface was different from that at the glass interface. It is the purpose of the present paper to describe the characteristic designs of growth at the fluid interface of culture of these cell strains, as seen between September 4, 1943, and January 4, 1945, supplemented by occasional visual and photographic observation through December 1946.

MATERIALS AND METHODS

The methods used in the maintenance of the cultures have been described (*1*). These were continued except for minor facilitating changes not influencing the results. In addition to these minor changes, however, in early May 1944 a defect developed in the temperature control system of the incubator containing the cultures. This resulted in the cultures being overheated to a maximum of 41.5° C. The extent and duration of this overheating are shown in the transcription of the temperature record chart of the incubator (text-fig. 1).

All cell descriptions are from visual examinations of the cultures and from detailed comparison of routine photographic records of the living cultures made over the period of study. To insure photographs in which the characteristics of all cell strains at both the glass and fluid interface could be compared at any one time, cultures of all strains were photographed on the same day, so that in any one group of cultures experimental conditions at the time photographed were as nearly identical as possible. This was made possible by the specially designed camera already described (*2*) which in this study has proved sufficiently flexible that up to 125 photographs could be made in a single work day. Where the number of cultures carried was so great as to make the photography of every culture impracticable, alternate culture or some comparable group of cultures selected by systematic sampling was used for the photographic records. To insure photographs characteristic of the respective culture strains and comparable with other photographs over a period of years, an attempt has been made to standardize the conditions further by recording the culture at a culture age of from 8 to 12 days and on the day following renewal of the culture fluid. Cultures were maintained at 37° C during photography and were handled with a minimum of shaking and exposure to light. Microscope light source was the Bausch and Lomb ribbon filament incandescent lamp, with Corning heat filter No. 397–2 mm. and deep yellow filter No. 352–2 mm. Practically all photographs were made on 8 by 10 inch Eastman Contrast Process Panchromatic film developed to high contrast by processing for 8 to 10 minutes at 72° F. in Eastman DK 76 fortified with 30 gm. of Kodalk per liter. Photographs were examined as negatives on an illuminated viewing unit which allowed simultaneous comparison of 90 8-by-10-inch negatives.

The magnification used was uniformly 200 ×. This film size and magnification allowed inclusion of a culture area of approximately 1 by 1.25 mm.



TEXT-FIGURE 1.—Linear transcription of the temperature curve recorded on circular chart of culture incubator thermograph from May 2 to May 15, 1944.

and at the same time recorded considerable intracellular detail. All photographs printed in this paper are areas from contact prints from such negatives. The total number of photographs compared has substantially exceeded 1,000.

RESULTS

General Observations

There was no constancy in the relative extent of the cell sheet at the fluid and glass layers. Some cultures showed growth limited to one interface or the other; other cultures had extensive cell sheets at both interfaces. In some instances a sheet of cells started out in one layer, and then, probably due to some tension or defect in the clot, turned, grew through the clot, reached the other interface, and there assumed the morphology characteristic of the interface it had reached.

Description of Cells

Strain D: Growth at the fluid interface was looser than at the glass. Mitoses were numerous. Except at the extreme periphery of the cell sheet, the cells were fairly uniform in size, were spindle shaped and radially oriented relative to the explant (fig. 1). Even when very close to each other, due to cell crowding, the cells showed almost no tendency to cohere laterally. At the extreme periphery of the culture, the cells were still usually spindle shaped, with occasional triangular or more sheetlike forms and tended to be more randomly oriented. Cell size in this zone showed greater variability, with occasional cells much larger than normal. In some cases they contained more than one nucleus (fig. 1). In this extreme peripheral zone occasional loose cells were seen, often with no evidence of having contact with the cell sheet.

Strain H: The general morphology of the cell sheet at the fluid interface of cultures of strain H was similar to that described for strain D. The sheet was still made up of radially oriented cells (fig. 2). The cells showed probably an even greater tendency than in strain D to remain laterally isolated. In the peripheral zone, there was the same tendency toward cell disorientation and production of abnormally large, more sheetlike cells as was observed in strain D.

Strain J: The cells at the fluid layer of strain J (fig. 3) were not definitely distinguishable from those of strain H, in either shape or in orientation. The cell sheet was possibly slightly looser than in strain H, while the separation and disorientation of cells at the periphery appeared even more severe than in strain H. An occasional peripherally situated larger cell was seen, like those shown in strain H. With this exception cell size was reasonably uniform. No appreciable increase in granulation was observed.

Strain L: The orientation, the shape, the granulation, and the lack of lateral cohesiveness of the cells of the fluid interface of strain L cultures were not definitely distinguishable from those of the fluid interface of strains D, H, and J. In one or two cases the morphology suggested that to be described in strain N cultures. The outstanding characteristic of

the strain L cultures was the presence of numerous abnormally large cells, some of which were extremely large.

Figure 4 shows a peripheral area of a strain L culture photographed January 4, 1945. The area was deliberately selected relatively free from very large cells in order to show the general morphology of the cell sheet. Even here one large cell is seen. Figures 4, 5, and 6 show a number of the very large cells.

Strain N: The morphology of the cell sheet at the fluid interface of strain N could be distinguished from that of any other strain, due to the characteristic, clearly defined, lateral separation of the spindle-shaped, radially oriented cells (fig. 7). Even when massed very close together (fig. 8), the cells were so laterally discrete as to actually appear laterally rounded. The cells within the sheets of these strain N cultures were extremely uniform in size (fig. 8), although occasional large cells were seen at the periphery of the cultures (fig. 9). This general uniformity of cell size distinguished this strain sharply from strain L. Cell granulation was definitely increased over that in strain L.

Strain O: The structure of the cell sheet at the fluid interface of strain O was easily distinguished from that of strain N and other culture strains. The cell sheet was no longer made up of a thin layer of laterally discrete cells; instead, the inner zone of the sheet was unusually thick, and while the orientation of its cells was in general radial the close massing of the cells and the extreme cell granulation largely concealed cell boundaries and shapes. In its more peripheral part, the sheet was broken up into rather short, loose, stringy strands of granular cells, which were loosely coherent laterally (fig. 10). The cells often appeared laterally shrunken. In numerous instances terminal cell processes were distally attached to other cell masses, were extremely tenuous, and suggestive of having been spun out as the cell masses had shrunken away from each other. In some extreme instances the cells tended to become spherical. Except for cell granules, the cells were often of such low refractive index as to be difficult to photograph satisfactorily. This morphology was substantially uniform from September 4, 1943, through December 19, 1946.

DISCUSSION

With respect to the influence of the overheating of the cultures in May 1944, although no cultures were killed, all were obviously injured. Growth practically ceased, and plans were made to close the study, but by the end of 60 days the cultures seemed to have recovered. Study of the cell strains was therefore continued. The later growth of the cultures was excellent and essentially comparable to that before overheating, while the general morphology at the fluid interface appeared unchanged. It appears that this period of overheating may be dismissed as having no appreciable role in permanently altering the cell design at the fluid interface.

In the present study, an attempt has been made to maintain a control and standardization of culture conditions such as composition of culture media, uniformity of transfer interval, uniformity of culture handling, and of temperature control. In making observations on the cultures, the age of the culture has been approximately standardized, and the zone and interface of the culture have been identified. With present day tissue culture procedures applied over such an extended period of time as is involved in this group of studies, such standardization has been practicable to only a limited degree. It is our observation, however, that as more accurate control over the variables of tissue-culture procedure has been obtained, the morphology of the cultures has shown a correspondingly increased reproducibility. It appears probable that even further advance may be made in the accuracy of morphologic studies of cells *in vitro* by further increased accuracy of standardization of the culture conditions used.

Detailed study of the cell design at the fluid interface was begun only after the cell design at the glass interface was well developed. Within the period studied there was no evidence of continued progressive change in the morphology of the cells at the fluid interface, nor was there any evidence of reversion to a less altered type in any strain.

These observations on the design of cells at the fluid interface, when taken alone, must be considered as of limited significance; however, when considered together with description of cell design at the glass interface, as already given and to be amplified in a later paper, they do assist in clarifying the design of cell changes that has taken place in these studies. A more detailed consideration of these changes and their relationship to the carcinogenic process will be given in later papers as other phases of the data are developed.

SUMMARY

The description of the cell design in the 6 sarcomatous cell strains previously described as having been originated from one strain of mouse subcutaneous connective tissue is continued. Attention is directed to characterization of cells lying in the plasma clot directly adjacent to the fluid culture medium.

In control strains D, never deliberately treated with carcinogen, and in strains H and J, treated for 6 and 32 days respectively with 20-methylcholanthrene, the cells at this fluid interface were generally radially oriented relative to the explant, loosely arranged, and, except at the extreme culture periphery, extremely uniform in size. Strain L treated 111 days, however, showed numerous abnormally large cells; strain N, treated 184 days, showed a characteristic lateral rounding of the cells; while strain O, treated 406 days, showed cells that were very granular and badly shrunken laterally.

Detailed study of the cell design at the fluid interface was begun only after the cell design at the glass interface was well developed. Within

the period studied there was no evidence of continued progressive change in the morphology of the cells at the fluid interface, nor was there any evidence of reversion to a less altered type in any strain.

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(*2*) ———: Production of malignancy in vitro. II. Photomicrographic equipment. J. Nat. Cancer Inst. 4: 135–145 (1943).

PLATE 128

FIGURE 1 (upper).—Cells of strain D photographed January 6, 1944, 9 days after planting. Note the lack of lateral cell cohesion in the radially oriented cells and the larger, more sheetlike cells at the periphery of the culture.

FIGURE 2 (lower).—Cells of strain H photographed November 3, 1943, 12 days after planting. Note the lack of lateral cohesion and the general radial orientation of the cells, except at the extreme periphery where the cells are more randomly oriented, more sheetlike, and larger.

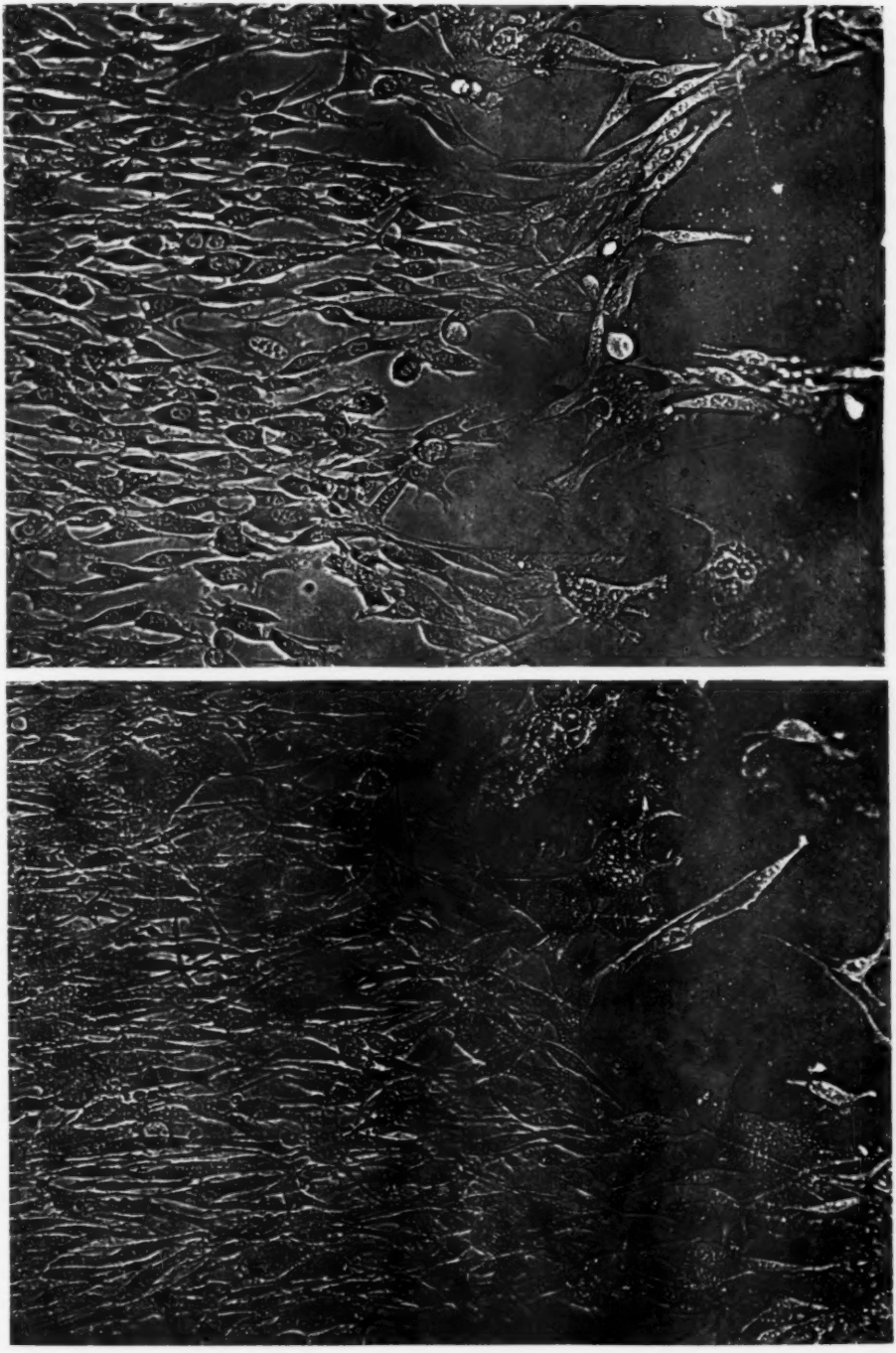


PLATE 129

FIGURE 3 (upper).—Cells of strain J photographed December 27, 1944, 9 days after planting. The cells within the culture sheet show less radial orientation than in Figures 1 and 2. The lighting in this photograph is somewhat oblique.

FIGURE 4 (lower).—Cells of strain L photographed January 4, 1945, 12 days after planting. The cells are still generally radially oriented relative to the explant. One very large sheetlike cell is shown at the lower right.

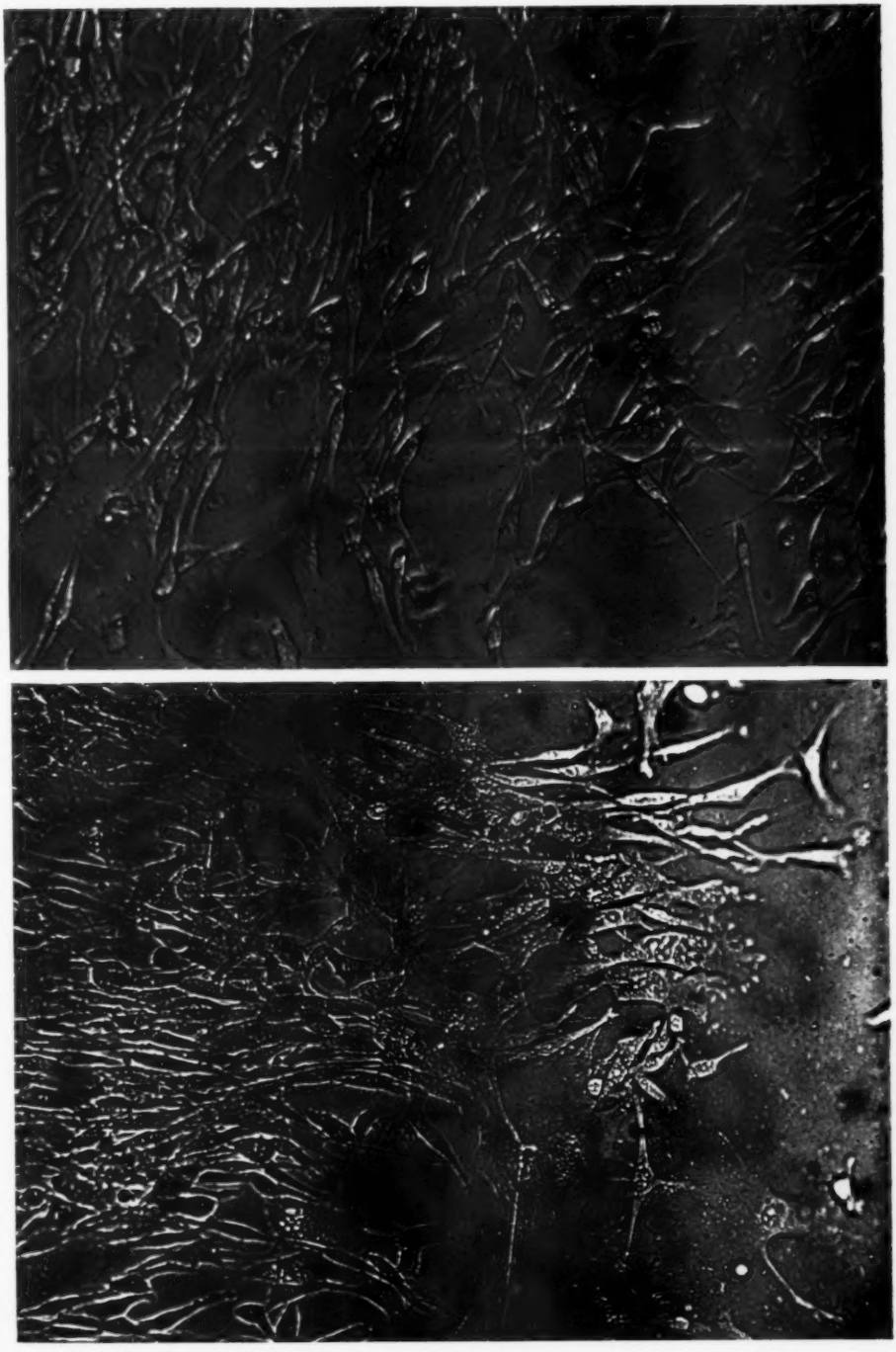


PLATE 130

FIGURE 5 (upper).—Cells of strain L culture photographed December 27, 1944, 9 days after planting. Note the design of the large peripheral cells.

FIGURE 6 (lower).—Cells of strain L culture photographed January 4, 1945, 12 days after planting. The limits of one large cell are approximately shown by four white dots. × 200.

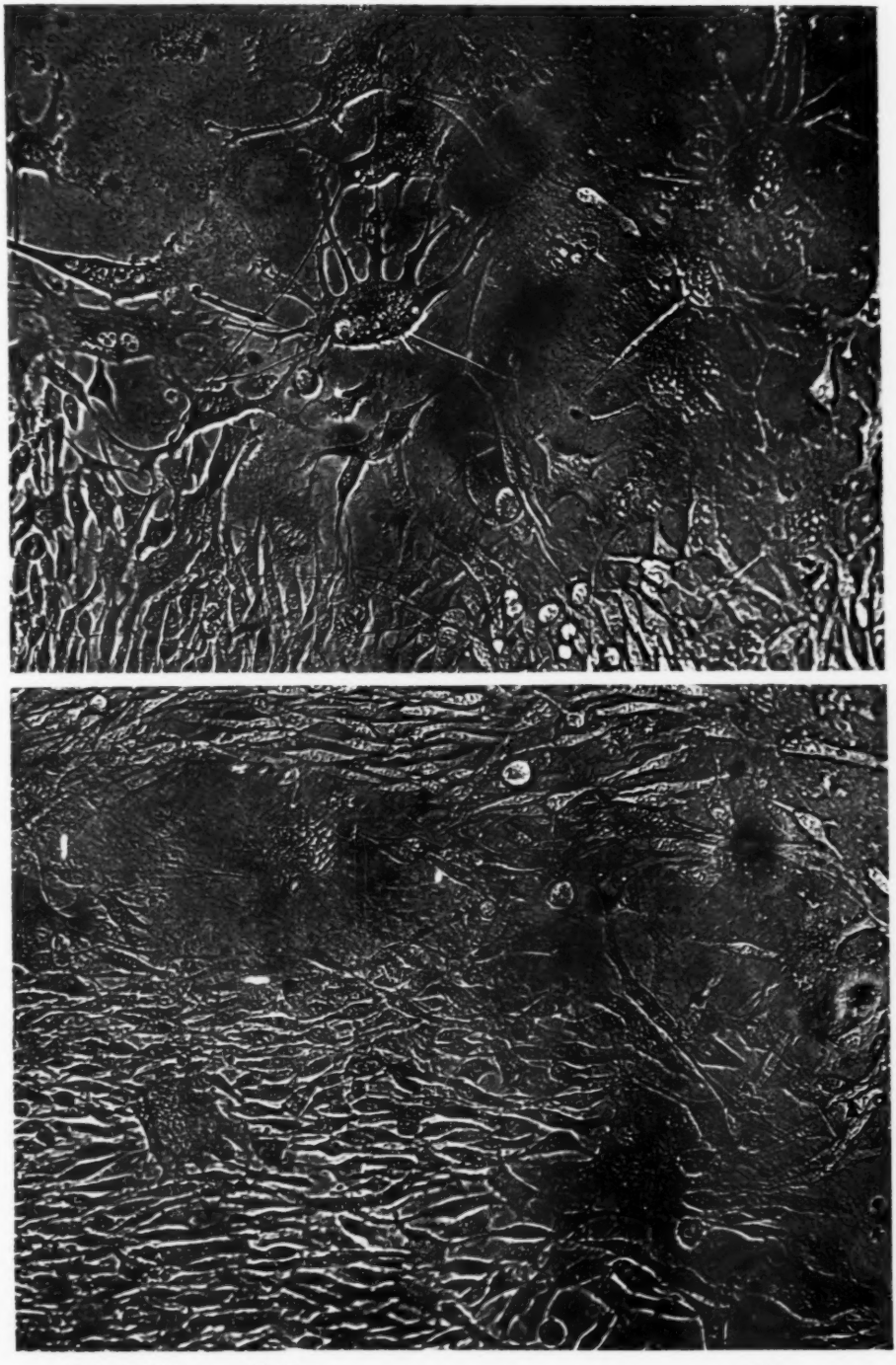
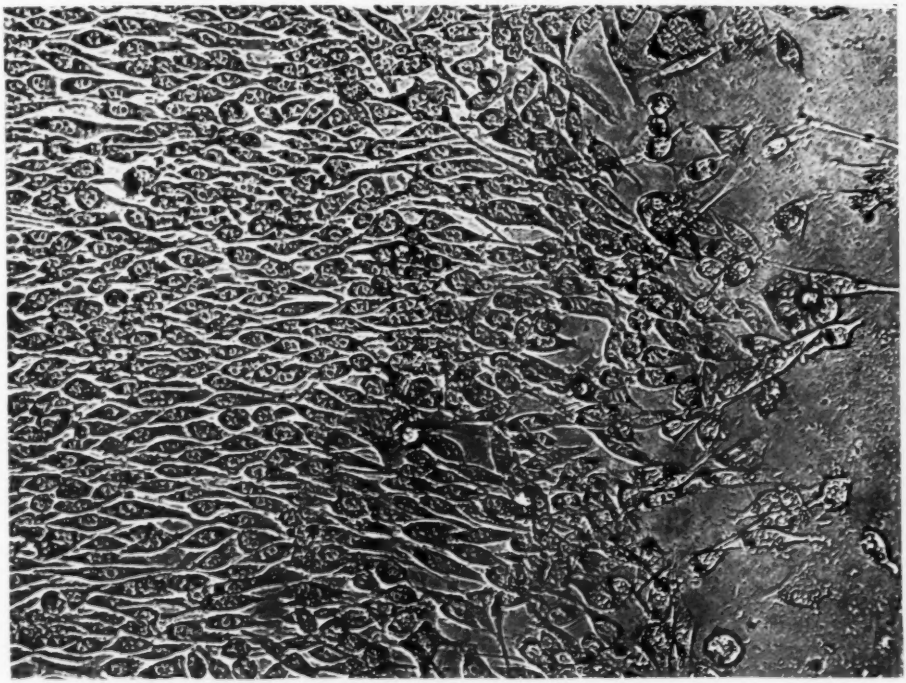


PLATE 131

FIGURE 7 (upper).—Cells of strain N culture photographed December 29, 1944, 10 days after planting. Note the characteristic appearance of lateral rounding and lateral discreteness of the cells.

FIGURE 8 (lower).—Cells of strain N photographed December 27, 1944, 9 days after planting. The area is slightly in from the culture periphery. Note the granulation of the cells and their tendency toward rounding.



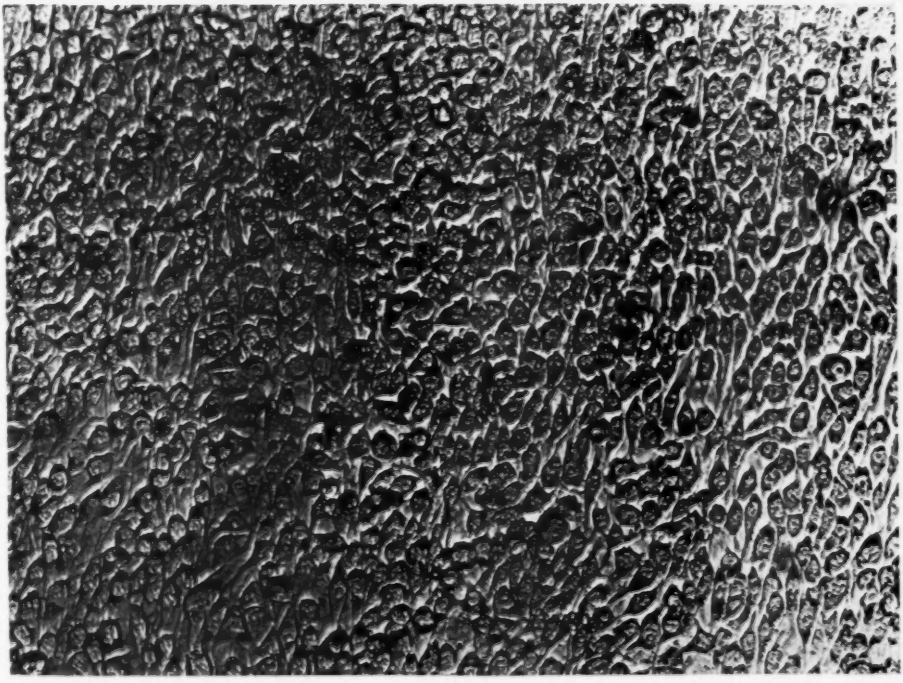
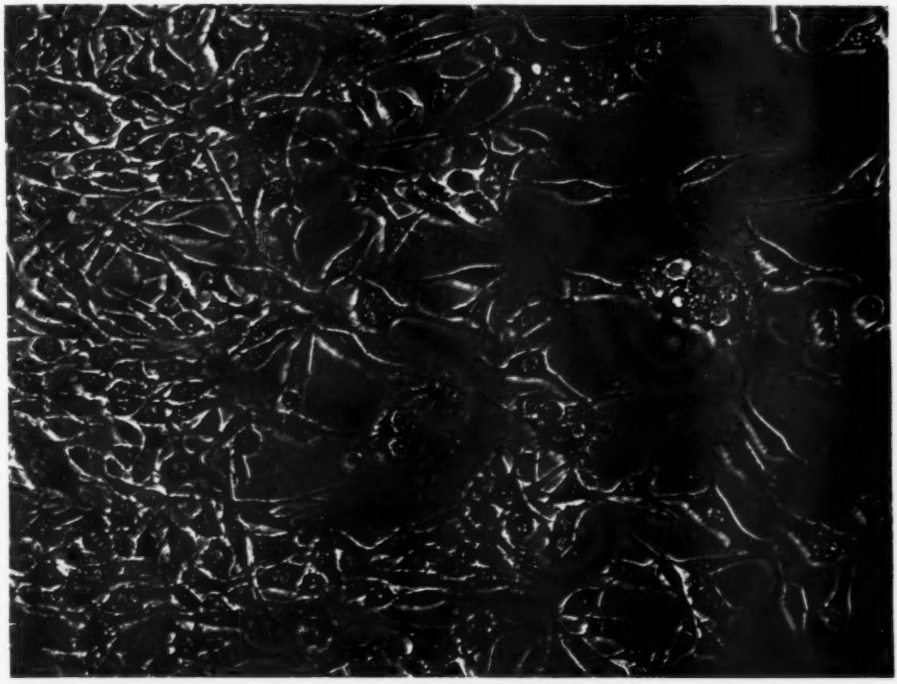


PLATE 132

FIGURE 9 (upper).—Cells of strain N photographed December 29, 1944, 10 days after planting. Note the severe granulation and the multiple nuclei of some of the large cells.

FIGURE 10 (lower).—Cells of strain O photographed January 4, 1945, 12 days after planting. Note the granular, stringy appearance of the cells.



USE OF PERFORATED CELLOPHANE SUBSTRATE IN SLIDE PREPARATION TISSUE CULTURES [1]

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In a previous publication Evans and Earle [2] described a procedure for growing certain normal and sarcomatous cells under sheets of perforated cellophane contained in Carrel D 3.5 or D 5.0 flasks.

Routine Carrel-flask cultures growing under cellophane can be studied satisfactorily with apochromatic microscope objectives of 16 mm. or with achromatic objectives of 8 mm., or even 5.5 or 4 mm. (N. A. .65) equivalent focus. When it is necessary to use objectives of shorter free-working distance than these recourse must usually be made to cultures planted as hollow-ground slide preparations.

In the present study an attempt is made to evaluate the practicability of using perforated cellophane sheets instead of plasma in such slide cultures.

MATERIALS AND METHODS

The growth of cultures in double coverslip slide preparations under a number of different experimental conditions has been compared. In the first type the explant was planted in a plasma matrix on the glass inner coverslip. Cultures of this type were considered as a control for those of the second and third types. In the second type culture, the explant was grown on a glass inner coverslip in a fluid culture medium and was covered by a disk of perforated cellophane placed on it. In the third type the inner glass coverslip was substituted by a disk of unperforated cellophane, a cell clump was placed on this, immersed in the fluid culture medium, and covered by a disk of perforated cellophane.

Pyrex hollow-ground slides used were 80 by 54 by 6 mm. in size, each having a central cavity, ground to a spherical curve, 40 mm. in diameter and 4 mm. deep. Before use these slides were cleaned with soap and water, then rinsed in distilled water.

Outer coverslips used were No. 1 thickness, 48 mm. square; inner glass coverslips were No. 1 thickness, 30 mm. circular. Both were cleaned in 2-percent-hydrochloric-acid alcohol, then dried on butter cloth.

[1] Received for publication October 10, 1949.

[2] Evans, Virginia J., and Earle, Wilton R. The use of perforated cellophane for the growth of cells in tissue culture. *J. Nat. Cancer Inst. 8:* 103–119 (1947).

Cellophane used was DuPont PT–62, thickness 450. The perforated cellophane was Beckley Pattern O design;[3] holes 0.023-inch diameter, straight line centers, 576 holes per square inch. All cellophane was cut into 28 mm.-diameter circles, then cleaned by giving three 10-minute changes each of ether, acetone, alcohol, and triple distilled water. The cellophane disks were then dried at room temperature between sheets of filter paper, placed in a petri dish, autoclaved for 1 hour, and then dried at 100° C. in the opened autoclave for 2 hours.

In planting the plasma cultures, a 30-mm. round coverslip was laid on a 48 mm. square coverslip and attached thereto by means of a small drop of fluid culture medium. One drop of plasma and one drop of a 50-percent mixture of horse serum and 1:1 chick-embryo extract from 9-day chicks were placed on the 30-mm. coverslip and spread over its surface to within about 1 mm. of its edge. The explant was then oriented in the culture medium, and the whole preparation was sealed by covering it with a hollow-ground slide carrying a ring of vaseline around its concavity. The preparation was finally given an outer seal of paraffin and incubated, coverslip down, at 37.5° C.

Where a perforated cellophane slip was used instead of the plasma, it was found that because of the absorbency of the cellophane the total volume of culture medium used could be increased without spilling over the edge of the round coverslip. In these preparations, therefore, three drops of the horse serum-chick embryo extract medium were placed on the 30-mm. circular inner glass coverslip. The explant was placed in this, and a 28-mm.-diameter disk of perforated cellophane lowered on top of it, after which the preparation was sealed as described. In preparations where unperforated cellophane was substituted for the glass round inner coverslip, the unperforated cellophane disk of 28 mm. diameter was attached to the square coverslip as described for the glass inner coverslip, after which the fluid medium and explant were added. The perforated cellophane disk was then lowered over the explant and the preparation sealed as described. Because of the increased absorbency of the cellophane, the total volume of fluid in these double cellophane slip preparations could be increased to 5 drops.

Tissues used in this study included heart, intestine, brain, skin, and choroid of the eye, all from 10-day chick embryos, and strain L mouse sarcoma cells, taken from stock plasma cultures maintained in this laboratory since 1940. All the chick tissues were used as freshly prepared explants, and these were cut to a size of about 1 by 2 by 3 mm. The strain L sarcoma culture explants were cut from rapidly growing 10-day plasma culture, the explants each being about 1 by 5 mm. in size. Four sets of cultures were studied, each consisting of about 9 cultures from each of these tissues. In studying these preparations little attention was given to identification and characterization of cells seen, interest being centered solely on evaluating the usefulness of the culture preparation.

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[3] Beckley Perforating Co., Garwood, N. J. Perforated cellophane is now available through the Tissue Culture Commission Testing Laboratory, Sloan-Kettering Institute, 444 East 68th St., New York, N. Y.

The culture medium on the slide preparations was renewed every 48 hours. To do this the paraffin seal was broken, and the square outer coverslip was lifted off and placed on the black glass planting plate. In the case of the plasma cultures, the inner circular coverslip was then removed with sterile forceps and placed in a sterile 5-inch petri dish. Two drops of fresh culture fluid were put on the growing cultures for 5 minutes. The slip was then placed on a new 48 by 48 mm. outer coverslip and remounted in the same way as had been done during the planting procedure.

In the case of preparations grown on either a glass coverslip or a slip of unperforated cellophane and covered with perforated cellophane, the coverslips underlying and overlying the preparations were lifted as a unit from the square outer coverslip and were blotted edgewise on sterile filter paper. Each such cellophane sandwich, the explant contained within it, was then placed on a new 48-mm. square outer coverslip together with two drops of fresh fluid medium. These preparations were then sealed on new depression slides.

All cultures were photographed at from 4 to 7 days of age. Photographs were routinely made at 200 × on Eastman Process Panchromatic cut film 8 by 10 inch size, and developed for 8 minutes at 72° F. in developer DK 76 containing 30 gm. of Kodalk per liter. Photographs are all areas cut from prints made from these negatives.

RESULTS

Practically all cultures of such tissues as chick heart or strain L sarcoma showed excellent migration, while skin, choroid, brain, and intestines were increasingly less constant in the order named. Initial migration appeared slightly earlier in the plasma cultures. No single type of preparation showed any clearly defined superiority in the cell migration seen, although there was a suggestion that migration in cellophane preparation was better. It did appear, however, that the cellophane preparation, particularly the preparation containing a double cellophane slip, remained in better condition over a longer period of days than did the plasma culture. This was almost certainly due to the larger amounts of culture medium that it was possible to use on the cellophane preparation.

Photographs of certain of these preparations are shown in figures 1–4.

The optical quality of all types of preparations was satisfactory, even when preparations were examined under high magnifications. One group of preparations was examined particularly critically with a 3 mm., 1.4 N. A. Bausch & Lomb apochromatic objective having a rated free working distance of 0.12 mm. In the plasma preparations, the cells lying within the plasma and close to the glass coverslip could be easily observed, but in the deeper layers of the plasma clot the objective was resting against the coverslip. The cells at and near the air interface of the plasma could not be sharply focused.

In preparations where an inner glass coverslip was used, and the cells were covered by perforated cellophane, the cells lying anywhere within the

preparation except at the free outer surface of the perforated cellophane interface could be sharply focused. These could not be seen because the objective was resting against the coverslip, but this limitation was of minor significance since it was rare that cells lay this close to the air interface of the culture. With double cellophane preparations, however, all depths of the preparations could be examined with ease. Even when the objective was focused on the air interface of the preparation, there was still considerable clearance between the front of the objective and the upper surface of the coverslip.

It was recognized that when cellophane is soaked in fluid, it swells somewhat. In order to obtain more accurate figures on the actual thickness of the various types of preparations, a number of preparations of each type was sacrificed and the actual thickness of the various components determined by means of a screw micrometer.

Whereas the glass coverslip was approximately 150μ in thickness, the thickness of the cellophane was only about 40μ. Consequently in those preparations made with an inner and outer glass coverslip and with plasma, the actual thickness of the preparation to the cell layer adjacent to the glass was at least 300μ. In preparations made with only a glass outer coverslip and with 2 cellophane sheets, the total overall thickness to the most distal cellophane sheet surface was only 230μ.

With both types of cellophane preparations it was found entirely practicable to open the preparation, remove the two inner slips with the cell clumps contained between them, and transfer them to a new slide preparation.

DISCUSSION

While it is generally recognized that for long-term studies the slide preparation is less easily handled than the culture flask or tube, the slide preparation has never been superseded where high magnification of the culture is necessary. In this role in particular, it appears from these data that the substitution of plasma by perforated cellophane, or even better, the substitution of plasma by perforated cellophane and the substitution of the glass inner coverslip by unperforated cellophane, has definite advantages. Insofar as is indicated by the scope of studies on these short-term cultures, it appears that such cellophane preparations are in a number of ways substantially superior to conventional plasma cultures. This superiority was particularly marked in the amount of culture fluid that could be used and in the added ease with which objectives of very short free working distance may be used.

While it appears that this 450 thickness of cellophane allows a preparation sufficiently thin for use with any lenses now in current usage, if the need arose even thinner preparations could be obtained by use of a No. 0 thickness outer coverslip and by using 300 thickness cellophane instead of that used in this study.

SUMMARY AND CONCLUSION

A study has been made of the practicability of using cellophane in double coverslip slide preparations of tissue culture. Types of preparations studied were (1) double glass coverslip preparations with the cells planted in plasma; (2) double glass coverslip preparations with the cells covered by a slip of perforated cellophane; (3) double coverslip preparation in which the inner slip was unperforated cellophane on which the cells were placed and covered by a second cellophane slip of perforated cellophane.

Tissues studied were heart, intestine, choroid, brain, and skin from 10-day chick embryos and explants from cultures of strain L mouse sarcoma.

All three types of preparations showed satisfactory cell migration. While the cellophane preparations were possibly slightly slower in starting migration, they were ultimately at least the equal of the plasma preparations.

The absorbency of the culture fluid by the cellophane permitted a larger volume of culture medium to be used in the cellophane culture than in the plasma, with the result that the cultures remained in better condition over a longer interval of time.

The cellophane slip cultures could be transferred from slide preparation to slide preparation as easily as could the glass coverslip plasma cultures.

Optical characteristics of the cellophane preparations were entirely satisfactory, while the extremely thin cellophane sheets allowed all levels of the culture to be examined with even a 3 mm. 1.4 apochromatic objective having a free working distance of 0.12 mm.

The conclusion is reached that the double cellophane slip preparation in particular has advantages that recommend its use in tissue-culture slide preparations. The trial of even thinner cellophane sheets is recommended in those cases where an extremely thin preparation is desired.

PLATE 133

FIGURE 1 (upper).—Four-day old culture chick heart, in double cellophane preparation. × 200.

FIGURE 2 (lower).—Four-day old culture of cells from the choroid of the eye, on glass coverslip covered with perforated cellophane. × 200.

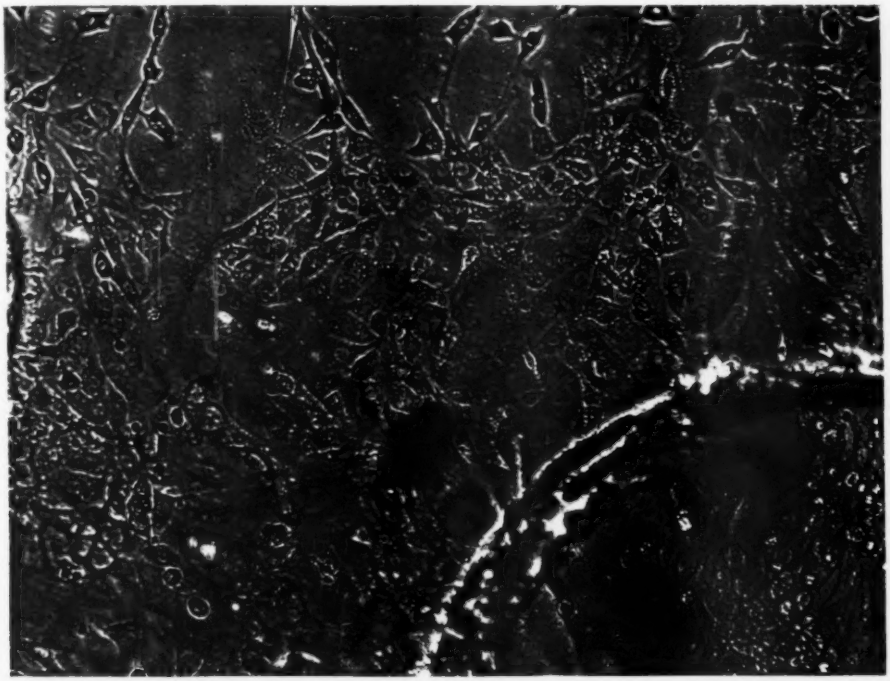
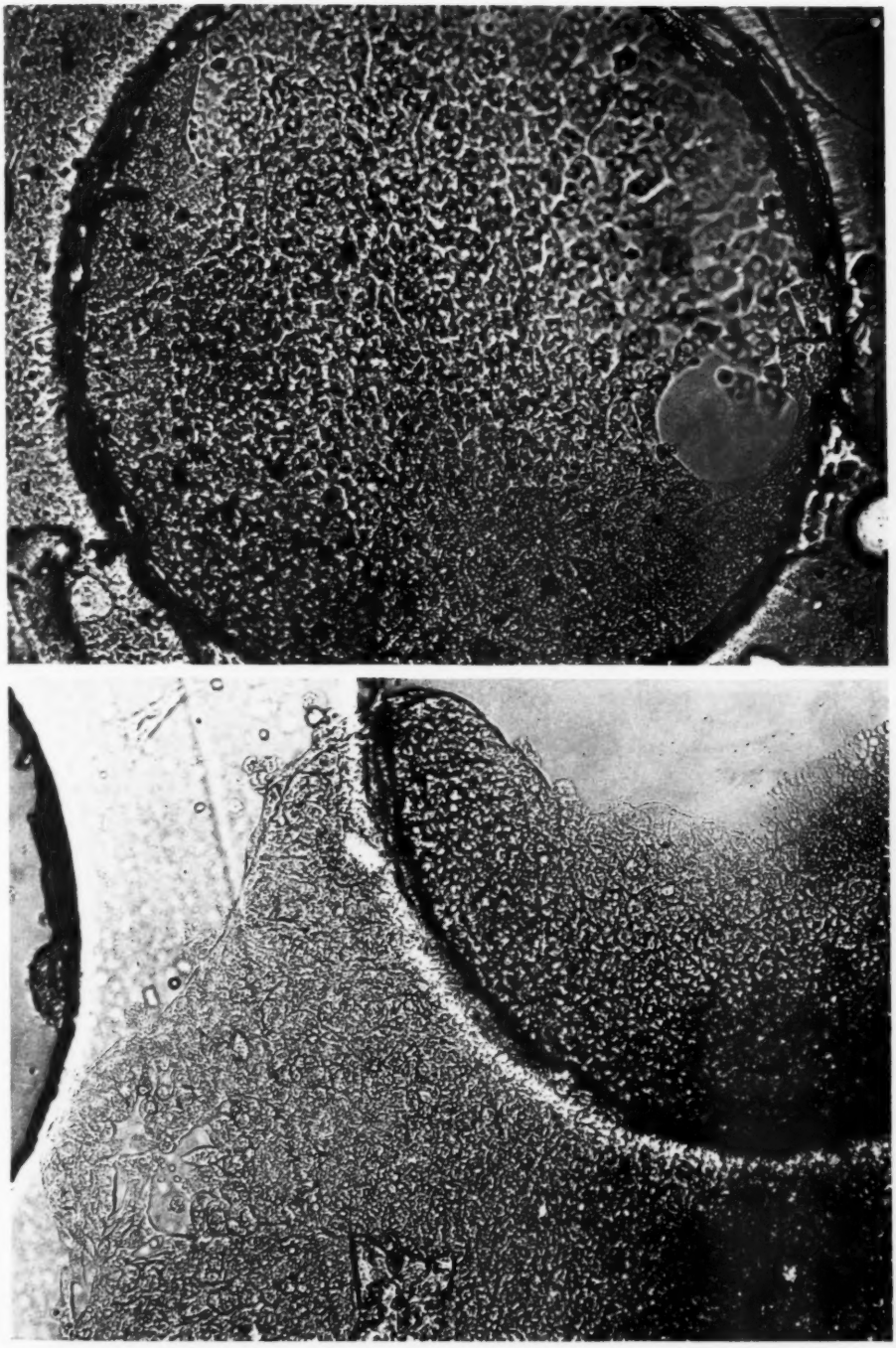


PLATE 134

FIGURE 3 (upper).—Five-day old culture of cells from the skin, on glass coverslip covered with perforated cellophane. The circular structure is one of the perforation through the cellophane sheet. × 200.

FIGURE 4 (lower).—Six-day old culture of cells from the central nervous system. × 200.



NOTE ON THE LACK OF PROGRESSIVE GROWTH OF METASTATIC MAMMARY CARCINOMA TRANSPLANTED SUBCUTANEOUSLY INTO FOREIGN STRAINS OF MICE [1]

RICHMOND T. PREHN, *senior assistant surgeon, National Cancer Institute, National Institutes of Health, Bethesda, Md.*[2]

The technique of intraocular transplantation was applied by Greene and Newton (*1*) to the study of the evolution of cancer in rabbits. They stated:

> With completed development, dependence ceases and the growth becomes an autonomous tumor or a cancer.
>
> Superficially such results and conclusions appear to be in disagreement with those obtained in mouse-tumor transplant experiments. The disagreement, however, is not actual but stems from differences in material, methods, and definitions. The transplantation of a spontaneous mouse tumor is generally effected from tissue obtained after the animal has been killed rather than from biopsy material. Moreover, transfer is usually performed when the tumor is first discovered rather than at the end of its developmental course. . .
>
> The transplantability of spontaneous mouse tumors has been under study in this laboratory using the same methods employed in the present investigation, and the results are essentially the same. The autologous transfer of early mouse tumors or the homologous transfer to members of the strain of origin is invariably successful, whereas transfer to alien strains or to guinea pigs fails. On the other hand, after complete development as determined by metastasis, transplantation to alien strains or to guinea pigs is readily effected (*1*).

Upon reading the above paragraphs, especially the last sentence with the word "readily," it occurred to this author that a metastatic nodule arising in a mouse of an inbred strain might grow progressively when implanted subcutaneously into mice from other inbred strains. The following experiments were designed to test this idea.

The mammary carcinomas used arose in mice of the C+ and C3H strains raised at this Institute (*2*, *3*).

The animals were kept until moribund in order to insure the greatest possible tumor development, then killed by cervical dislocation and examined for lung metastases. Only those animals bearing metastases large enough for both histologic section and transplantation were used. In some cases the primary tumors and in others the metastases were transplanted.

Transplantations into the axillae of the test mice were done with a sterile 12-gauge trocar.

[1] Received for publication November 7, 1949.

[2] The author wishes to express his thanks to Dr. H. B. Andervont in whose laboratory this work was performed.

The test animals were from the inbred strains C, C3H, C57 black, and I. They were equally divided as to sex; their ages ranged from 8 to 370 days.

Metastases (or primary tumors that had metastasized) from 13 mice were transplanted to 76 test and 17 control animals as follows:

Seven metastatic C+ tumors were transplanted into:
- 13 C3H mice.
- 11 C57 black mice.
- 7C mice (control).

Four metastatic C3H tumors were transplanted into:
- 12 C57 black mice.
- 15 C mice.
- 3 I mice.
- 7 C3H mice (control).

One metastatic C X C3H F_1 hybrid tumor was transplanted into:
- 4 C3H mice.
- 4 C57 black mice.
- 4 C mice.
- 3 C X C3H F_1 hybrid mice (control).

One metastatic C X C3H F_1 hybrid tumor was transplanted into:
- 2 C3H mice.
- 4 C57 black mice.
- 4 C mice.

The test animals were kept for observation for from 4 to 6 months following inoculation.

In only one case did the transplanted material grow progressively and continuously in a test mouse. In that case the tumor arising in a C X C3H F_1 hybrid grew in one out of four male test C mice. The transplant lingered as a small stationary red nodule for two months. It then ulcerated and began a slow growth. The animal was killed four months after inoculation, at which time the transplant was an ulcerated, necrotic mass, 15 by 15 by 10 mm. Microscopic section revealed tumor tissue resembling the original transplant material.

In a few cases small masses (1–5 mm.) were palpable at the site of inoculation up to the third to fifth day, after which they disappeared. These swellings may have been foreign body reactions or tumor growths, but no sections were made at this stage.

The transplants invariably grew and killed the hosts in the control animals of the same strain from which the transplant material was obtained.

The results of these limited experiments showed that, according to the procedures used, primary mouse mammary tumors that had metastasized (or their metastases) very rarely demonstrated the ability to grow progressively when transplanted subcutaneously into foreign strains of mice.

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(*3*) ———: Spontaneous tumors in a subline of strain C3H mice. J. Nat. Cancer Inst. 1: 737–744 (1941).

RESPONSE OF MAMMARY-TUMOR-AGENT-FREE STRAIN DBA FEMALE MICE TO PERCUTANEOUS APPLICATION OF METHYLCHOLANTHRENE [1]

HOWARD B. ANDERVONT, *biologist*, and THELMA B. DUNN, *pathologist, National Cancer Institute, National Institutes of Health, Public Health Service, Bethesda, Md.*

Engelbreth-Holm (*1*) was the first to discover that percutaneous application of methylcholanthrene to strain dba mice accelerated the development of mammary-gland tumors. Nineteen females 4 to 6 weeks old were painted 23 times at weekly intervals with a 0.5 percent solution of the carcinogen in benzene and all developed mammary tumors. Seventeen developed multiple mammary tumors; a total of 58 mammary carcinomas arose in the 19 painted mice. One animal also had leukemia.

He emphasized the difference between his findings and those of Morton and Mider (*2*) and Mider and Morton (*3*) who applied the carcinogen to the skin of dba mice and found that the development of leukemia was accelerated. These investigators started painting when the mice were 4 to 5 weeks old and applied a 0.5 percent solution of methylcholanthrene in benzene twice weekly until death of the animals. Of 16 females 14 developed general or mediastinal lymphomatosis and only 2 of these had mammary carcinomas. Of 22 males painted by Engelbreth-Holm none developed leukemia, and of 25 painted by Mider and Morton 22 developed either general or mediastinal lymphomatosis.

These paradoxical results cannot be explained by reading the publications of the investigators. Engelbreth-Holm derived his mice from animals procured from the Roscoe B. Jackson Memorial Laboratory and found that 50 percent of breeding females developed mammary tumors when over 9 months of age: the incidence of spontaneous leukemia was 2 percent in both sexes when over 6 months of age. Mider and Morton obtained their animals directly from the same source and were informed that leukemia occurred spontaneously in both sexes between the ages of 650 to 800 days. This suggests a subline difference in susceptibility between the experimental animals used by the Danish and American investigators. However, in a later paper Morton and Mider (*4*) state that while their previous experiment was performed with a "mixture of various lines of the strain," they repeated their procedures with litter

[1] Received for publication November 17, 1949.

mates of "line 212" raised in their laboratory and of 156 mice of both sexes, 150 developed general lymphomatosis and 4 solitary mediastinal lymphoma. They mentioned the occurrence of breast carcinomas by stating that this type of tumor was associated with general lymphomatosis: the number of mice with mammary tumors was not given. In their hands subline 212 was a low spontaneous leukemia strain.

The only specific difference mentioned by these investigators was dosage of the carcinogen. All used a 0.5 percent solution of methylcholanthrene in benzene. Engelbreth-Holm, however, gave 23 weekly paintings while Mider and Morton (*3*) painted twice weekly until death of the mice and Morton and Mider (*4*) presumably employed the same technique, although the authors did not give the precise technique of application in their latter paper.

Kirschbaum, Lawrason, Kaplan and Bittner (*5*) noted the striking difference between the observations of the above-mentioned investigators and suggested that the discrepancy may have been the result of using different sublines of dba mice or different experimental procedures. They observed that Morton and Mider used nonbreeding females, whereas Engelbreth-Holm did not mention whether his females were kept as breeders or nonbreeders. This apparently suggested to Kirschbaum *et al.* that breeding may exert some influence on the development of carcinogen-induced mammary tumors in dba mice. Consequently, they painted 6-to-8-weeks-old dba (subline 12) mice twice weekly with a 0.5 percent solution of methylcholanthrene in benzene, the duration of treatment was not given. Twenty-seven breeding females all developed mammary cancer at an average age of 155 days, and of 31 virgin females 4 developed mammary cancers at an average "induction time" of 191 days. Of 26 untreated subline 12 breeding females, 23 developed mammary cancer at an average age of 370 days. The authors stated, "the incidence of mammary cancer in virgin females of subline 12 has not been determined." Thus, their findings showed clearly that application of the carcinogen accelerated the appearance of mammary tumors in breeding females of the subline but did not produce any information concerning its influence in virgins.

Kirschbaum *et al.* also found that leukemia occurred in their methylcholanthrene-painted dba mice of both sexes and suggested that some of the virgin females may have failed to develop mammary tumors because of the earlier occurrence of leukemia. They mentioned that none of the untreated control breeders developed leukemia. This implies that, in common with Engelbreth-Holm and Morton and Mider, their experimental animals were of a low leukemia subline of strain dba.

Another common characteristic of the sublines of dba mice used in the three laboratories was their susceptibility to the development of spontaneous mammary tumors, and the investigations of Engelbreth-Holm and Kirschbaum *et al.* showed clearly that in their sublines, percutaneous

administration of methylcholanthrene accelerated the appearance of these tumors. Since strain dba is a high-breast-tumor strain (*6*) and is known to possess the mouse mammary tumor-agent, this acceleration of mammary-tumor development occurred in the presence of the agent.

This paper is a report of four experiments in which agent-free strain dba mice were given percutaneous applications of methylcholanthrene. The primary objective of the study was to ascertain whether the carcinogen was capable of inducing mammary tumors in agent-free strain dba females. If the animals developed mammary tumors then answers could be sought to the following questions:

1. Would the agent and methylcholanthrene reveal synergistic activity in the production of tumors?
2. Would the mammary tumor-agent be present in the induced tumors or in the tumor-bearing animals?
3. Would the histologic appearance of the methylcholanthrene-induced mammary tumors differ from those usually found in high-breast-tumor strains of mice?

The investigations provided evidence that mammary tumors were evoked in the agent-free females and the tumors and tumor-bearing mice were deficient in the agent. They also revealed that mammary tumors induced by the carcinogen showed a variety of histologic types whereas those developing in the presence of the agent showed a more uniform histologic pattern.

MATERIALS AND METHODS

Female mice from an agent-free line of strain dba were used. The establishment of this line was described previously (*7*). It was derived from a colony of strain dba mice (substrain 212) showing an incidence of 43 percent mammary tumors at an average age of 15 months in breeding females. A litter was removed from the uterus of a colony female and suckled by an agent-free strain C3H female. This litter contained one female which was bred to her brother; all members of the agent-free line were descendants of these mice. Virtually all agent-free females cast from 2 to 5 litters and brother-to-sister matings have produced 7 completed generations containing 174 females that lived to a mean age of 20 months and, of these, only 1 developed a mammary tumor. Precise information of the occurrence of spontaneous leukemia in breeding females is not available but the incidence is less than 10 percent.[2]

Two procedures were used to test for the agent in methylcholanthrene-treated dba mice. In some, one gram of tumor tissue was minced with scissors, ground thoroughly with sand, and diluted in 5 cc. of distilled water. After standing for a few minutes the supernatant fluid was in-

[2] Dr. W. E. Heston of the National Cancer Institute has kindly supplied the following information concerning his experience with subline 212 dba mice. In 1944, 50 virgin females were set aside in an attempt to determine the incidence of spontaneous leukemia for the strain and 19, or 39 percent, developed spontaneous leukemia at an average age of 17.3 months. The nonleukemic mice died at an average age of 19.7 months.

jected intraperitoneally into test mice. Each mouse received 0.1 cc. or 0.2 cc. of extract. In others, pieces of the spleen and tumor from tumor-bearing mice were implanted subcutaneously into the test mice. All tissue implants were made subcutaneously in the right axillary region.

Mice used as test animals for detection of the mammary tumor-agent were members of inbred strains, or their hybrids, which were known to be susceptible to the agent. These mice were 7 to 21 days old when they received subcutaneous or peritoneal inoculations of test substances. They were mated when 2 months old, and each produced one litter which was removed soon after birth. They were observed for the appearance of tumors until at least 18 months of age.

All mice were kept eight in a cage and were examined every other week for mammary tumors. They were maintained under similar environmental conditions, with an unlimited supply of tap water and Purina laboratory chow pellets.

Stilbestrol was used to produce estrogenic stimulation in some animals. Pellets containing 10 percent stilbestrol dissolved in cholesterol were prepared according to the technique described by Shimkin and White (*8*). Each pellet weighed 2.5 to 3.0 mg. and was inserted subcutaneously into the right axilla. All pellets used in these experiments were from a lot prepared before the start of the investigations. Only those animals in which the pellets were found at autopsy were included in the results.

Methylcholanthrene (m. p. 179°–180° C.), hereafter referred to as MCA, was dissolved in reagent (thiophene free) benzene (Merck) and applied to the unshaven skin by a single upward stroke of a No. 6 camel-hair brush. In order to avoid as far as possible the production of skin tumors, the carcinogen was not applied continuously to the same region but to seven different sites. These, in order of rotation were: the lateral surface of the right front, left front and right hind legs, the sacral, abdominal and scapular regions and the lateral surface of the left hind leg. An adequate supply of MCA in benzene was prepared for each experiment and kept in a glass-stoppered container in the dark. An amount sufficient for one application to the animals of each experiment was removed from the container at each painting. The concentration of carcinogen and the number of applications are given in the description of individual experiments.

Extracts of lactating mammary glands served as a source of mammary tumor-agent. Nursing females from strains C and C3H carrying the agent were removed from their litters on the tenth day of lactation. Sixteen hours later they were killed and their mammary glands removed and squeezed in a tissue press. The mixture of milk and tissue extract was strained through several layers of fine gauze, diluted with distilled water, and 0.1 cc. administered intraperitoneally to young agent-free dba females 7 to 14 days of age. The concentration of the extract is given in the individual experiments.

EXPERIMENTAL PROCEDURES AND RESULTS

Experiment 1

This experiment was designed to supply an answer to each of the three questions raised in the introduction. Females from 39 litters of agent-free dba mice were used. When the mice were 7 to 14 days old, half of each litter received a 1:1 dilution of extract of lactating mammary glands which contained the agent. When the last litters were weaned, all the mice that had received the agent and half of the remainder were each given a stilbestrol-cholesterol pellet. They were from 1 to 3 months of age at that time. Stilbestrol was administered because Kirschbaum *et al.* (*5*) had shown that following percutaneous application of MCA to dba mice, the breeding females showed a higher incidence of mammary tumors than did virgin animals.

Percutaneous application of the carcinogen was started the day following insertion of pellets. Half of the mice that had received the agent and stilbestrol pellet, and all of those that had not received the agent, were painted. This produced four groups of mice arranged so that, when possible, each litter contained a representative of each group. The first group received the agent, stilbestrol, and MCA; the second received the agent and stilbestrol; the third stilbestrol and MCA; the fourth MCA only. The last two groups were used to ascertain whether estrogenic stimulation was essential to produce mammary tumors in these agent-free dba females, and, if the mammary tumor agent was present in the MCA-induced tumors, whether estrogenic stimulation enhanced its activity.

A 0.5 percent solution of MCA was used and was applied twice weekly for a total of 28 paintings.

The results are summarized in table 1. Many of the MCA-treated animals developed leukemia and skin tumors. Indeed, the high incidences of leukemia at an early age in the MCA-treated animals was the outstanding result of the experiment.

Virtually all leukemias were lymphocytic in type and were divided into three groups according to the sites of hypertrophy of lymphatic tissue. Localized masses in the anterior mediastinum (thymoma), resulting in sudden death, predominated in the animals which were the first to show macroscopic lesions. The second type was characterized by involvement of the mediastinal nodes and a few subcutaneous or peritoneal nodes. These animals survived longer, on the average, than those with mediastinal lesions only. The third type prevailed in those animals that lived to a relatively late average age. It consisted of generalized hypertrophy involving the axillary, inguinal, and cervical lymph nodes and, in addition, some of the mesenteric and retroperitoneal nodes. Usually there was no macroscopic mediastinal involvement in this type of lesion.

Many MCA-treated mice also developed tumors of the skin. The figures in this column of table 1 include papillomas and carcinomas and are

TABLE 1.—*Summary of four experiments to study induction of mammary gland tumors in strain dba females*

Experiment No.	Experimental procedure	Amount of MCA in benzene	Number of paintings weekly	Total number of paintings	Age of mice at start of paintings	Number of mice	Response													
							Mammary gland tumors			Type and number of mammary gland tumors					Leukemia		Skin tumors		No tumors	
							Number of mice	Tumor incidence	Average age	Adenocarcinoma	Adenoacanthoma	Carcinosarcoma	Sarcoma	Miscellaneous	Number of mice	Average age	Number of mice	Average age	Number of mice	Average age at death
		Percent			*Months*			*Percent*	*Months*							*Months*		*Months*		*Months*
1	Agent + Stilbestrol + MCA.[1]	0. 5	2	28	1 to 3	19	4	21	9	3	----	----	1	----	13	7	10	6	1	8
1	Agent + Stilbestrol	----	----	----	1 to 3	16	11	69	13	11	----	----	----	----	0	----	0	----	5	12
1	Stilbestrol + MCA	. 5	2	28	1 to 3	14	1	7	7	1	----	----	----	----	12	6	10	6	1	9
1	MCA	. 5	2	28	1 to 3	14	4	29	8	3	----	1	----	----	10	6	10	6	0	----
2	Stilbestrol + MCA	. 5	2	37	6 to 9	20	13	65	12	5	4	4	1	1	5	11	12	12	0	----
2	MCA	. 5	2	37	6 to 9	23	17	74	12	5	5	5	----	3	5	12	13	12	0	----
3	Stilbestrol + MCA	. 25	1	21	2 to 5	45	18	40	13	5	9	4	----	1	1	8	25	12	4	14
3	MCA	. 25	1	21	2 to 5	42	20	47	12	7	8	3	2	2	1	14	18	13	4	13
4	Agent + MCA	. 25	1	14	1 to 5	32	26	81	10	22	2	----	----	2	1	9	1	12	4	12
4	Agent	----	----	----	1 to 5	33	29	88	11	29	----	----	----	----	0	----	0	----	4	11
4	MCA	. 25	1	14	1 to 5	39	12	31	14	4	4	3	1	----	1	12	10	14	15	12

[1] MCA denotes methylcholanthrene.

not accurate for this experiment because the occurrence of skin tumors in these mice was not recorded carefully. All mice autopsied when more than 6 months old had developed multiple papillomas, many of which were removed from the sites of application of the MCA.[3]

This experiment showed that in these dba females, 28 percutaneous applications twice weekly of a 0.5 percent solution of MCA in benzene induced many leukemias and skin tumors.

Experiment 2

This experiment was performed concurrently with experiment 1. Availability of a group of virgin agent-free dba females which were 6 to 9 months old, led to an attempt to ascertain whether percutaneous administration of MCA would induce mammary tumors. The animals belonged to 23 litters and half of each litter received a stilbestrol-cholesterol pellet. Four days later all received the first painting with the same solution of carcinogen used in experiment 1. The carcinogen was applied twice weekly for a total of 37 applications.

The response of these animals is shown in table 1. Of 43 mice, 30 developed mammary gland tumors and those exposed to MCA alone showed a higher incidence than litter mates that received both stilbestrol and MCA. Apparently stilbestrol did not increase the susceptibility of these mice to the induction of mammary tumors following percutaneous application of the carcinogen.

TYPES OF MAMMARY GLAND TUMORS

The occurrence of mammary tumors in the mice of this experiment showed that the carcinogen had induced these tumors in agent-free dba females. In conformity with previously stated objectives of the experiments a study of the histologic appearance of the induced neoplasms was made and a description of the types of mammary gland tumors encountered in this and other experiments of this series is presented at this point.

Histologic sections were prepared from all palpable tumors beneath the skin arising in the dba mice. The tissues were fixed in Tellyesnicsky's fluid (2 parts formol, 20 parts 70 percent alcohol and 1 part acetic acid) blocked in paraffin, cut, and stained with hematoxylin and eosin.

The tumors were examined histologically and sorted according to the criteria described. A preliminary statement on the peculiarities of morphology of mammary tumors in mice is required. There is often a great dissimilarity between individual tumors, yet the group as a whole is sufficiently distinctive so that one has little difficulty in identification. The variability indeed may be considered a distinguishing characteristic, for, unlike lung tumors, these tumors do not exhibit a uniform histologic

[3] A group of 20 strain C females 1 to 3 months of age were painted with the same solution of MCA and at the same time as were the dba mice of this experiment. Ten received stilbestrol pellets and MCA and 10 MCA alone. None developed a mammary tumor or leukemia. Ten developed skin tumors at a much later average age than did the dba mice. This observation indicated a pronounced difference in the degree of susceptibility between the two strains. Morton and Mider (4) obtained similar results with the same strains.

pattern, but instead usually vary histologically from area to area. Serial sections would be required to catalogue all the morphologic patterns which may appear in a single tumor, and different tumors from the same animal also frequently differ as to the prevailing pattern. Even on transplantation complete uniformity of pattern is not acquired, and the changes developing in transplanted lines attracted the interest of early workers in the field who described them minutely.

Despite this general lack of uniformity, the majority of these tumors still show a restricted range of modification from an easily recognized basic acinar structure, which is seen especially in mice with the tumor agent. Although many elaborate classifications have been devised for these tumors, the statement was made in an earlier publication (*9*) that no good purpose was served by an attempt to classify them precisely and that the designation "mammary tumor" was sufficient. A similar opinion has been expressed by Willis (*10*) regarding breast tumors in the human female. He writes, "Quite commonly different parts of one tumor show different structural variants, and terms such as 'comedo', 'scirrhous', 'medullary', 'adeno', and 'simplex' have only locally descriptive, not classificatory value." He further states that histologic subdivisions of mammary carcinoma are arbitrary, and that these types of growth are not entities but merely variants of the entity, mammary carcinoma.

Nevertheless, while we still believe that an attempt to make a rigid histopathologic classification of the mammary tumors in mice would be wasted effort, when the tumors in the present experiments were examined, it was noted that many of them arising in mice without the tumor agent fell outside the range of morphologic variation generally encountered in mice with the agent. It was suspected, therefore, that an attempt at grouping them might reveal a significant preponderance of some histologic variant. Such a grouping is warranted when a large number of tumors are to be examined belonging to a uniform animal source, and when these tumors can be compared with other large uniform collections. It is not to be thought that each tumor fits into an exact category, for different observers would probably arrive at slightly different results in sorting these tumors, certainly if different sections were taken for study. On the other hand, a definite trend of morphologic variation becomes apparent, and one variant may be sufficiently preponderant in the mice included in one experiment so that a real difference is disclosed.

When the tumors from the strain dba mice in the present experiments were reviewed, we found that they could be placed in the following groups:

1. Adenocarcinoma.—We are using the term adenocarcinoma to designate tumors with an obvious glandular structure and representing the typical or characteristic morphology of these tumors. It is the group on which Apolant (*11*) based his elaborate description and classification. We have found that tumors of this form preponderate in mice with the mammary tumor-agent. Usually the tumors are composed of large areas consisting of small glandular or tubular structures lined by cuboidal epithelial cells in a rather compact arrangement which suggest a reproduction of the mammary acini (fig. 1*A*). The gland lumens may be very minute and empty or filled with small amounts of secretion, resembling thyroid follicles. Many modifications of this basic

structure may occur. Cyst formation, with papillary ingrowth is common (fig. 1*B*). The stroma may be delicate and edematous or thick and hyalinized, and differences in the aggregation of the acini, which may be closely grouped, scattered, or gathered into bands, may occur. In spite of the great individual diversity, the group as a whole appears surprisingly uniform. They may be considered as covering a fixed range, and are readily identified as mammary tumors. These tumors grow readily when transplanted, metastasize, and kill the host, so that they are malignant. It is our opinion that any separation of mammary tumors in mice into benign and malignant on morphologic grounds is artificial, since it is not reflected in biological behavior.

2. Adenoacanthoma.—Small scattered patches of squamous cells are not uncommon in many mammary tumors, but in the tumors grouped here, large areas, sometimes nearly the entire cross section, showed an epidermoid structure (fig. 2*A*). Such tumors have been referred to as "keratinized tumors," and the epidermoid areas as "squamous cell metaplasia." This epidermoid formation, however, is in many instances an integral part of the tumor development, and the structure resembles the adenoacanthomas described in human beings. Indications of malignancy such as an increased number of mitoses and anaplastic structure can be seen in the squamous epithelium (fig. 2*B*) and metastases and transplantation usually reproduce this epidermoid element. This structure, therefore, cannot be considered as a nonmalignant secondary transformation of glandular tissue in the same sense as squamous-cell metaplasia in bronchiectasis or other inflammatory processes. In a previous report (*9*), we found such tumors in about 10 percent of mice from a collection of inbred strains. Recent workers (*12*) have reported them as especially frequent in animals without the mammary tumor agent. A similar increased incidence was evident in the agent-free strain dba mice of the present experiments in which they occurred in about one-third of the animals. Among these tumors were three examples of the so-called molluscoid type which was first described by Haaland (*13*). He observed that this tumor was composed of lobes radiating like the spokes of a wheel from a central core of squamous epidermoid tissue. The neoplasm suggests a balanced malignant transformation of several elements inherent in the gland, perhaps indicating an attempted reproduction of an organ rather than a single tissue (figs. 3*A* and *B*).

3. Carcinosarcoma.—This tumor was remarkably frequent in MCA-painted strain dba mice without the agent. Primary tumors of this type have been described in non-inbred mice (*14*) and we have seen it rarely in mice of inbred strains at the National Cancer Institute. On serial transplantation of the adenocarcinomas, mixed tumors such as these not infrequently appear in early generations to be succeeded in later generations by uniformly "sarcomatous" tumors such as the well known S 37. The high incidence of primary carcinosarcomas in these strain dba mice is, therefore, of considerable interest because of this "sarcoma" transformation and because of the pure sarcomas of the mammary gland area, to be described in the next paragraph. Some examples of this type exhibit a blending of round epithelial cells and elongate spindle cells resembling fibroblasts (fig. 4*A*). Both elements may show numerous mitotic figures, and both may appear malignant (fig. 4*B*). In some areas the epithelial cells appear to be assuming a spindle form (fig. 4*A*). Whether this represents anaplasia in carcinoma cells, or whether a malignant change occurs in the stromal cells, either independently or under the influence of the growing carcinoma, has been the subject of much speculation and study and it is still undecided. These tumors sometimes resemble the desmoplastic carcinomas seen in human beings, and transitions from the characteristic epithelial mammary tumor of the mouse, with an excessive stromal formation, to the unmistakable carcinosarcoma were seen in these strain dba mice. It was often difficult to decide whether to put a particular tumor into the adenocarcinoma or the carcinosarcoma group because of this gradual transition. In one tumor (fig. 4*C*) the epithelial and sarcomatous elements are sharply demarcated suggesting a development from separate foci.

4. Sarcoma.—Such tumors in the mammary-gland area have been previously described (*15*). They are composed of interlacing bundles of spindle cells, and are

similar to sarcomas originating at other sites except that the spindle cells envelop the mammary ducts (figs. 5*A* and *B*). We believe that this tumor should be reported here along with other mammary tumors because of its probable origin from mammary connective tissue. Tumor giant cells are not infrequent (fig. 5*B*). The histogenesis of these sarcomas may be closely related to the carcinosarcomas, since transition stages can be found. Since they occurred only in animals receiving MCA, this may be of particular significance as an etiologic factor in this group of tumors.

5. Miscellaneous.—This is the most ill-defined collection. It represents a group of tumors that cannot be placed in any of the other categories described here, yet they do not form a uniform type among themselves. Many of the tumors from these strain dba mice which we placed in this miscellaneous group resembled adenocarcinomas in the "typical" group except that the amount of stroma was so abundant as to place them outside the range of modification due to stromal increase which may be found in tumors of groups of mice with the tumor agent. In addition to this form several rare types of mammary tumor were placed in this miscellaneous group. Such tumors merit detailed individual descriptions beyond the scope of the present paper. One with areas of dissociated cells is shown (fig. 6*A*). Another showed what may be myoepithelial elements (fig. 6*B*). Each of these tumors could still be recognized as of mammary origin, because some areas were unmistakable and showed the usual acinar structure.

Distribution of mammary tumors according to types is shown in table 1. The difference between the number of tumors diagnosed and the number of tumor-bearing mice was due to the presence of 2 mammary tumors in a few mice: these tumors were not always of the same histologic type. Only 10 tumors were adenocarcinomas. The most interesting feature of the histologic diagnosis was the relatively large number of adenoacanthomas and carcinosarcomas. This showed that the carcinogen had induced different types of mammary gland tumors in this line of agent-free dba mice.

Two tumors were tested for the agent. Both arose in mice exposed to MCA alone and were diagnosed as typical mammary tumors or adenocarcinomas. Extracts of these tumors were administered to 22 test mice which lived to a mean age of 19 months and none developed a mammary tumor.

Ten animals developed leukemia of which 7 had mediastinal tumors only. The relatively low incidence of leukemia indicated that the 6-to-9-months-old mice of this experiment were more resistant to this type of induced neoplasm than were the 1-to-3-months-old animals of the first experiment. This increased resistance to induced leukemia probably enabled the mice to live long enough to develop mammary tumors.

Table 1 data show that 25 mice developed skin tumors. More of the animals had this type of tumor but, as in experiment 1, an accurate record of these tumors was not kept in this experiment.

The results of this investigation showed that the agent-free dba mice were susceptible to the induction of mammary gland tumors by MCA and that the carcinogen induced a variety of tumor types. It was also shown that the estrogenic stimulation supplied by the stilbestrol-cholesterol pellets did not enhance the effect of MCA in producing mammary gland tumors. The older mice of this experiment were more resistant to induced leukemia than were the younger animals of experiment 1.

Experiment 3

This experiment was started after experiments 1 and 2 were finished and the primary objective was to determine the amount of MCA necessary to produce mammary tumors in young dba mice. The outcome of experiment 1 showed that 28 percutaneous applications of a 0.5 percent concentration of the carcinogen was far above the desired amount.

Forty-one litters of mice were used. Half of each litter was given a stilbestrol pellet when 2 to 5 months of age and 15 days later skin painting was started. Each mouse received a weekly application of 0.25 percent of MCA in benzene for 21 consecutive weeks. Thus, each painting site received 3 applications of the carcinogen.

As shown in table 1, of 87 mice 38 developed tumors of the mammary gland and, as in experiment 2, stilbestrol pellets did not influence the incidence of these neoplasms. The table also shows that the carcinogen again produced a variety of tumor types: 12 were adenocarcinomas, 17 were adenoacanthomas, and 7 were carcinosarcomas.

The most interesting result was the sharp reduction in the number of mice developing leukemia when compared to those of experiment 1: only 2 of 87 mice in this experiment developed leukemia whereas of 28 comparable mice in experiment 1, 22 developed leukemia.

Forty-three mice developed skin tumors. Many of these were papillomas and were found in mice that had developed mammary tumors. But a sufficient number of mice developed skin carcinomas and died without mammary tumors to show that the dose of carcinogen was above the optimum amount necessary to induce mammary tumors only.

Eight mice were tested for the mammary tumor-agent by the implantation of pieces of their spleens and tumors [4] into test mice. Three (2 adenocarcinomas and 1 adenoacanthoma) arose in mice exposed to stilbestrol and MCA, and 5 (2 adenocarcinomas, 1 adenoacanthoma and 2 sarcomas) arose in mice exposed to MCA alone. A total of 38 test animals were used in these tests; they lived to a mean age of 18 months and none developed a mammary tumor.

The outcome of this experiment showed that mammary tumors were induced in young agent-free dba mice when an appropriate amount of MCA (21 weekly applications of 0.25 percent in benzene) was applied percutaneously. The dose of carcinogen used in this experiment induced leukemia in only 2 mice but induced skin tumors in many mice. Tests for the presence of the mammary tumor agent in the tumors and in tumor-bearing mice gave negative results.

Experiment 4

The primary purpose of this experiment was to ascertain whether measured amounts of the agent and carcinogen would exert a synergistic effect in producing mammary tumors in the agent-free dba mice. The

[4] Two of these tumors, both sarcomas, when transplanted into strain C females grew progressively and killed the hosts. This presented an opportunity to study tumors that arose in one inbred strain of mice and possessed the ability to grow in at least one other inbred strain. Furthermore, the tumors had never been transplanted into members of their strain of origin. These tumors are not included in the table.

procedure employed was determined by the outcome of the three previous investigations. The animals were not given stilbestrol pellets and the dose of MCA was reduced further.

Forty-four litters of mice were used. When the young were 7 to 14 days old 2 of every 3 females received a 1:10 dilution of extract of strain C3H lactating mammary glands containing the tumor agent. When the last litters were weaned, half the mice that had received the agent and all those that had not received the agent were painted once each week with a 0.25 percent solution of MCA for 14 weeks. The mice were from 1 to 5 months of age when the painting started; each site was given two applications of the carcinogen.

The results (table 1) showed that the mice exposed to both the agent and carcinogen as well as those exposed to the agent alone, showed high incidences of mammary tumors, whereas those that had received the carcinogen alone exhibited a relatively low incidence. Only 2 mice developed leukemia; both had general lymph node enlargement. One animal exposed to both agent and carcinogen developed a skin tumor, and 10 exposed to the carcinogen alone developed similar tumors. Most of these tumors were papillomas and were found in those mice autopsied late in the course of the experiment.

All mice that received the agent alone, and most of those exposed to the agent and carcinogen, developed adenocarcinomas (table 1). Of 39 animals exposed to MCA alone, 4 developed adenocarcinomas, 4 developed adenoacanthomas, and 3 had carcinosarcomas.

Four mice that received applications of the carcinogen alone were tested for the agent. All had developed mammary tumors; 2 were diagnosed as adenocarcinomas, 1 as an adenoacanthoma, and 1 as a carcinosarcoma. Both adenocarcinomas were used to prepare extracts: The other 2 tumor-bearing mice were tested by inoculation of pieces of their spleens and tumors into the test animals. A total of 22 animals received the tumor extracts and tissues: they are living at a mean age of 16 months and none has developed a mammary tumor.

The results of this experiment showed that a combination of agent and carcinogen did not accelerate the occurrence of mammary tumors. The carcinogen induced fewer mammary tumors than did the agent alone or the agent in combination with the carcinogen. The application of carcinogen in this experiment (14 weekly paintings of 0.25 percent in benzene) induced leukemia in only 2 mice and a relatively small number of skin tumors. Most of the skin tumors were papillomas.

Tests for the mammary tumor-agent in mice that were exposed to MCA have, thus far, given negative results.

DISCUSSION

This discussion includes only those publications dealing with the induction of mammary tumors, leukemia or skin tumors in strain dba mice by topical application of methylcholanthrene and is limited largely to those which may have a direct bearing upon the results presented in this paper.

Law (*16*) and Engelbreth-Holm and Lefèvre (*17*) induced mammary tumors, leukemia and skin tumors in dba mice by percutaneous and subcutaneous administration, respectively, of 9,10-dimethyl-1,2-benzanthracene. Their work showed that the production of these tumors in strain dba mice is not limited to methylcholanthrene. The reader is referred to the paper by Engelbreth-Holm and Lefèvre for a good review of the early literature concerning the acceleration of mammary tumors and leukemia in mice following application of coal tar or carcinogenic hydrocarbons. Strong and Smith (*18*), Bonser and Orr (*19*), Strong and Williams (*20*), and Strong (*21*) found that methylcholanthrene produced mammary tumors in other strains. Kirschbaum, Strong and Gardner (*22*), as well as Kirschbaum and Strong (*23*), found that the carcinogen produced leukemia in other strains. The paper by Kirschbaum and Strong contains pertinent references to the induction of leukemia in other strains of mice by other carcinogens. Finally, the investigations of Bonser (*24*), Strong and Williams (*20*), Orr (*25*), Strong (*21*), and of Kirschbaum and Bittner (*26*) showed that methylcholanthrene induced mammary tumors in inbred mice and their hybrids which were free of the mammary tumor-agent.

Strain dba (subline 212) mice used in the experiments reported herein were derived from one of the first inbred strains to be established and, according to recent information (*6*), the strain contains various sublines some of which are separated by 30 generations of inbreeding. This difference alone is sufficient to account for some of the divergent findings recorded in this discussion, and, when other influences such as environmental conditions of different laboratories, different diets and techniques are included, some discrepancies in results should be expected. Recognition of the importance of these factors is made at this time to avoid repeated references to them when the findings of various investigators are presented.

It is also important to note that throughout this paper the use of the terms "dose" and "dosage" when referring to percutaneous application of methylcholanthrene does not imply definite knowledge of the amount of carcinogen responsible for the induction of tumors. It is impossible to know the precise dosage of any carcinogen when it is applied topically to the unshaven skin in a volatile solvent. Furthermore, the licking habits of mice raise the question whether the mammary tumors and leukemias were induced by ingested carcinogen. Orr (*25*) found that intranasal administration of methylcholanthrene produced mammary tumors in mice.

Probably the most interesting result encountered in the present experiments was the difference in response of young agent-free female dba mice to varying doses of methylcholanthrene dissolved in benzene. Twenty-eight percutaneous applications twice weekly of a 0.5 percent concentration of the carcinogen induced leukemia and skin tumors in many mice and mammary tumors in only a few. Twenty-one weekly applications of a 0.25 percent concentration induced skin tumors and mammary tumors in approximately half the animals and only 2 cases of leukemia. Following 14 weekly paintings of a 0.25 percent concentration approximately one-

third of the mice developed mammary tumors, one-fourth developed skin tumors and 1 developed leukemia. This was a clear-cut demonstration of an inbred mouse strain developing different types of tumors in response to varying amounts of a carcinogen.

As stated earlier, dosage of the carcinogen could account for the different findings of Engelbreth-Holm (*1*) and Mider and Morton (*3*). The former gave 4-to-6-weeks-old mice 23 weekly paintings of a 0.5 percent concentration whereas the latter workers gave 4-to-5-weeks-old animals applications twice weekly of the same concentration until death of the animals. Virtually all Engelbreth-Holm's mice developed mammary cancer whereas those of Mider and Morton developed leukemia. Neither publication mentions the use of a particular subline of strain dba. In later investigations Morton and Mider (*4*) used sublines 212 and 122 and again their mice showed a high incidence of leukemia when painted with a 0.5 percent solution or a 0.25 percent solution of the carcinogen in benzene. The weaker concentration gave a longer latent period only. The number of applications was not given. However, in this paper they recorded the occurrence of mammary tumors in 5 of 22 subline 212 females painted with a 0.25 percent concentration of the carcinogen in acetone and while discussing this observation stated that breast carcinomas, in association with leukemia, also occurred in mice painted with benzene solutions. They did not give the number of such animals or the concentration of hydrocarbon used.

In a more recent series of publications Mider in cooperation with J. White and F. R. White studied the influence of diet upon the induction of leukemia in dba mice and, of immediate interest here, used small doses of the carcinogen. In all these reports the carcinogen was applied to the skin on alternate days as a 0.2 percent solution in ether, and young "weanling" litter mate dba mice were used. In the first report (*27*) 19 control mice of subline 212 (sex not specified) were painted until death [average of 44.7 paintings (*28*)] and 17 developed leukemia at a mean latent period of 81 days. In the second (*28*), (subline 212 mice, sex not specified) each of 40 control mice was painted 20 times and 36 developed leukemia with a mean latent period of 99 days. This paper is also of interest because it reports that, in preliminary experiments, dba mice were given 10, 15, 20 or 30 paintings and in those painted 20 times the incidence of leukemia approximated that obtained from 40 to 70 paintings. The third paper (*29*) records the use of both sexes (subline not given) and of 26 control mice painted 25 times, 25 developed leukemia with a mean latent period of 73 days. This paper is of special interest because the control mice developed leukemia only. Of 20 mice on a restricted diet, 4 developed epidermoid carcinoma of the skin, 2 developed papilloma of the skin and 1 a subcutaneous fibrosarcoma. The preceding papers in the series do not mention tumors other than leukemia in the control mice. In the last paper (*30*) both sexes (subline not given) were used. Ten diets were fed and 20 paintings were given. The results are presented here because 390 mice showed incidences of 27.5 percent to 92.1 percent

of leukemia with mean latent periods of 91 to 136 days, depending upon the diet the mice ingested. Other tumors are not mentioned.

According to their publications induced leukemia predominated in subline 212 mice after 20 to 25 percutaneous applications on alternate days of a 0.2 percent solution of carcinogen in ether. The total dose of carcinogen was comparable to that of experiments 3 and 4 reported here in which 126 subline 212 females received 14 or 21 weekly applications of a 0.25 percent solution in benzene and 50 developed mammary tumors, 53 developed skin tumors, and only 3 developed leukemia. The presence of mammary tumors in the agent-free mice of the present experiments and their absence in Mider's mice, which were carrying the agent, lend further emphasis to the discrepancy.

Two differences in techniques could account for the different results. Benzene and ether were used as solvents. The animals were painted at weekly intervals in the experiments reported here whereas they were painted on alternate days in Mider's experiments. Thus, the results suggest that the technique of application as well as dosage may be of importance in the response of mice to skin-painting with carcinogens.

Tannenbaum and Silverstone (*31*) observed a different response to small amounts of the carcinogen. They used 250 14-to-18-weeks-old dba males (subline not stated) and applied a 0.3 percent solution in acetone to the skin of the interscapular area by means of a dropping pipette. Each mouse received 5 drops at 5-day intervals followed by a 14-week interval of no applications and then 7 more drops at 5-day intervals. They record the appearance of skin tumors only: leukemia is not mentioned in their results.

This brief review of results obtained by other investigators who applied methylcholanthrene to the skin of dba mice suggests that either the genetic constitutions of the animals or the experimental procedures or both must have exerted a pronounced influence upon the ability of relatively small amounts of the carcinogen to elicit tumors. Other strains of inbred mice possess varying degrees of tissue susceptibilities to carcinogens. Strain A mice are probably the best example because their lungs are far more susceptible to the development of both spontaneous and hydrocarbon-induced tumors than are their other tissues (*32*). Administration of o-aminoazotoluene to strain C females induces hepatic and pulmonary tumors, hemangioendotheliomas at various sites, as well as sarcomas at the site of administration (*33*). The tissue response of this strain is not only dependent upon the dosage of the carcinogen but upon the site of administration as well as the solvent used (*34*). The observations recorded herein show that strain dba mice also possess varying degrees of tissue susceptibilities to methylcholanthrene-induced tumors and that their mammary gland tissues are susceptible to tumor induction by both methylcholanthrene and the mammary tumor-agent.

Another finding of interest in the present work was the influence of age upon the response of dba females to the carcinogen. In the first experiment, 1-to-3-months-old females received 28 applications of a 0.5 percent

concentration and many developed leukemia, whereas in experiment 2, 6-to-9-months-old females received 37 paintings with the same solution of carcinogen and many developed mammary tumors.

Morton and Mider (*4*) found that the latent period for induction of leukemia was longer in their dba mice that were over 80 days of age when painting was started. Law (*16*) observed that 2-to-16-months-old dba mice were more resistant than month-old mice to leukemia induced by percutaneous administration of 9, 10-dimethyl-1,2-benzanthracene. These investigators did not report the occurrence of a different type of tumor in the older mice. The dose of carcinogen they employed may have been so large that the mice died of leukemia before they could develop mammary tumors or the presence of the mammary tumor-agent in their experimental animals may have obscured their results. It is of interest to note that Kirschbaum *et al.* (*5*) recognized the possibility that some of their dba mice may have died of leukemia before they could develop induced mammary tumors.

Apparently the mice of experiments 1 and 2 revealed a higher incidence of localized mediastinal tumors than had been encountered previously. Mider and Morton (*3*) as well as Morton and Mider (*4*) found mediastinal lesions only in some of their animals, but in their subline 212 mice only 4 of 156 painted with a 0.5 percent solution of the carcinogen and only 2 of 44 painted with a 0.25 percent solution developed this form of lymphomatosis. Law (*16*) also found general involvement of the lymph nodes in his mice. Age, apparently, was not of much importance in these divergent findings because both Morton and Mider and Law started painting mice which were, on the average, younger than those of experiment 1. Furthermore, in experiment 2, of 10 older mice in which leukemia occurred, 7 had localized mediastinal tumors. This suggests that the prevalence of this form of lymphoma in the mice of the present experiments was the result of either subline susceptibility or dosage of carcinogen or of both.

The lack of uniformity in the results obtained by all investigators who administered methylcholanthrene to strain dba mice indicates that a large number of factors are involved in the occurrence of induced tumors when the carcinogen is painted on the skin.

The primary objective of the present studies was attained when mammary tumors were induced in the older agent-free dba mice of experiment 2. This result was confirmed in experiment 3, which was designed to ascertain the dosage of carcinogen necessary to induce this type of tumor in younger animals. In both experiments it was found that the occurrence of mammary tumors was not dependent upon estrogenic stimulation supplied by stilbestrol.

These findings made possible an attempt to ascertain whether measured amounts of the mammary tumor-agent and methylcholanthrene would reveal synergistic activity in the production of mammary tumors. In experiment 4 the mice receiving the agent alone showed a higher incidence of mammary tumors than did those receiving the agent and carcinogen.

This result is not interpreted as showing that the agent and carcinogen cannot act synergistically because the amount of agent used in experiment 4 may have been so large that it masked the influence of the carcinogen. Such investigations usually require the application of amounts of both substances which, when administered alone, will produce a small number of tumors. This was not the case in experiment 4. This problem is receiving further consideration.

Another objective of the studies was to determine whether the mammary tumor-agent was present in the carcinogen-induced tumors or in the tumor-bearing mice. The administration of extracts of carcinogen-induced tumors and of pieces of tumors and spleens from mice bearing tumors did not produce tumors in the test animals. Therefore, according to the techniques employed, the agent was not detected in these mice or tumors. This does not exclude the possibility that the agent may appear in an occasional mouse or that it was present in an attenuated form.

The occurrence of papillomas and carcinomas of the skin in the treated mice confirmed previous knowledge that the hydrocarbon induced this type of tumor. The prevalence of skin tumors in these experiments indicated that the mice were very susceptible to this type of neoplasm. Despite the rotation of painting sites and the use of small amounts of carcinogen in experiment 4 an appreciable number of these tumors appeared. Engelbreth-Holm (*1*) used the same technique of application for a stronger concentration of the carcinogen but did not obtain as many skin tumors. Mider and Morton (*3*) also rotated sites and painted until death of the mice but record a relatively small number of skin carcinomas. Morton and Mider (*4*) did not record the presence of skin tumors in their mice while White *et al.* mention the occurrence of these tumors in but one paper (*29*) of their series.

A study of the histologic structure of the induced mammary tumors was also included in the present investigations and proved to be of considerable interest. The types of tumors encountered are described herein. These consisted of the typical acinar type of adenocarcinoma usually seen in mice from high-mammary-tumor strains. This type of tumor arose in all mice that received the mammary tumor-agent alone and confirmed Bonser's (*35*) opinion that the agent produces a high degree of acinous hyperplasia. Four other types, designated as adenoacanthoma (or keratinized), carcinosarcoma, sarcoma, and miscellaneous, always arose in mice which had received percutaneous applications of methylcholanthrene. Many more of these four types were encountered in mice that had received the carcinogen alone than in those that had received both the carcinogen and the agent. This indicates that the histologic structure of the tumors was dependent upon their etiology. However, some of the carcinogen-induced tumors were indistinguishable from those in which the agent was involved.

Analysis of the carcinogen-induced mammary tumors revealed that all types arose in mice of the same average age.

Engelbreth-Holm (*1*) observed squamous-cell metaplasia in tumors

induced in dba mice by the carcinogen. One tumor had a "sarcoma-like stroma." Mider and Morton (*3*) found two fibrous type sarcomas in their dba mice. Strong and Williams (*20*) were apparently the first to direct attention to variations in the histologic structures of tumors induced by the carcinogen in the absence of the agent. They injected strain NH mice subcutaneously and described 45 mammary tumors of which 11 showed squamous metaplasia with marked keratinization equivalent in all probability to our adenoacanthoma and 5 were of "scirrhous type" which probably resembles our carcinosarcoma or the transition between the typical adenocarcinoma and this type. Strong (*21*) used strain NHO mice and further emphasized the difference between these tumors and those produced in the presence of the agent by describing four characteristics possessed by the carcinogen-induced tumors, namely, squamous metaplasia, pronounced anaplastic changes, anaplastic tumors involving surrounding normal tissues, and metastases to bone.

Kirschbaum and Bittner (*26*) record a "variety of histologic patterns" in carcinogen-induced breast tumors arising in hybrid mice free of the tumor agent. Their description of the tumors emphasized the presence of squamous metaplasia in all the induced growths and mentions a sarcomatous change in the stromal tissue of two. Later Kirschbaum, Williams and Bittner (*12*) published a more comprehensive account of their observations. They again described squamous metaplasia as characteristic of carcinogen-induced tumors and stated that this change was apparent even in the precancerous induced nodules. "These nodules, which developed in either the presence or absence of the milk agent, are thought to have been induced by the carcinogen." They regarded the nodules as precancerous lesions because "there was both gross and microscopic evidence for transformation to carcinoma."

A short time previous to the paper of Kirschbaum, Williams and Bittner, Huseby and Bittner (*36*) had published a thorough study of the histologic morphology of mammary glands of inbred mice and their hybrids to correlate the architecture of the glands and the factors necessary for the production of spontaneous mammary tumors. They did not induce mammary tumors in their animals by the administration of chemical carcinogens but described a lesion which appears to be identical to the nodules observed by Kirschbaum *et al.* This lesion was referred to as "nodules" and "these consist mainly of metaplasia of glandular to squamous-type epithelium." Huseby and Bittner characterized their lesion as inflammatory in nature and Kirschbaum *et al.* also described "an accompanying inflammatory cellular infiltration in the surrounding connective tissue" of their nodules. Huseby and Bittner observed the nodules with equal frequency in high and low tumor strains and concluded that they "did not appear to be precancerous in nature."

Later, Browning (*37*) observed similar lesions in the mammary glands of strain C3H mice and agreed with Huseby and Bittner's conclusion that they were not precancerous because they "did not progress further, or regressed and disappeared during gross observations." He made an

interesting contribution to the study of the lesion by showing that in C3H mice these nodules "never occurred at a larger size than 0.175 cm." whereas "no tumors of 0.2 cm. or over had this histology, and none were ever seen to regress." His criteria cannot be applied to the lesions described by the other investigators because neither Kirschbaum *et al.* nor Huseby and Bittner gave the precise size of their "nodules" although Kirschbaum *et al.* refer to "minute nodules." In an earlier paper Kirschbaum and Bittner (*26*) referred to "any lesion less than 3 mm. in diameter" as a nodule.

None of the tumors of the experiments described here were of such small size that they could be called nodules. The tumors of the first three experiments were not measured but most of those of experiment 4 were measured when the animals were necropsied. The smallest was 10 mm. in diameter and the largest was 20 mm. by 20 mm. by 15 mm. in size. Thus, the present studies contributed little information to the interesting problem of the histogenesis of chemically-induced and agent-induced mammary tumors.

Apparently, the strain dba mice of the present experiments were the first agent-free animals of this strain in which breast tumors were induced by the carcinogen and the histologic variations in these tumors exceeded those described by other investigators. The occurrence of the typical acinous type of tumor in all mice which received the agent alone, in most of those which had received both agent and carcinogen and in a relatively small number that had received carcinogen alone indicated that absence of the agent permitted the carcinogen to exert its influence upon a variety of mammary gland tissues.

It seems clear from the histologic examination of the tumors from these experiments that mice with the tumor agent were prone to develop adenocarcinoma with a basic acinar structure with only a limited variation from this pattern. In contrast mice without the agent exhibited a considerable increase in the incidence of more unusual forms of mammary tumor. One possible explanation for this difference is that several elements of the mouse mammary gland undergo neoplastic transformation singly or in combination. Certain evidence (*35*) suggests that the agent acts particularly on the acinous tissue of the gland, and initiates adenocarcinoma at an early age. If true, it can be expected that when the agent is present, mammary tumors of a predominantly acinar form develop at an early age and kill the animal before the more unusual types emerge. When the agent is absent, adenocarcinoma also develops, but in addition unusual forms of mammary tumor appear, particularly after treatment with a carcinogen. The data in the table support this view. Our experience with mice of other strains free from the agent suggests the possibility that different tumor types may differ in their relative incidence depending upon the strain of mouse. In these dba mice, a carcinosarcoma type was remarkably frequent, and the adenoacanthoma was above the expected incidence as compared with dba mice with the agent. Much more work will be required before strain differences can be determined posi-

tively, but it may explain the difference in some of the first reports appearing on the morphology of mammary tumors in mice without the agent.

SUMMARY AND CONCLUSIONS

Four experiments were performed in which mammary-tumor agent-free virgin female strain dba mice, subline 212, were given percutaneous applications of methylcholanthrene dissolved in benzene. In three experiments estrogenic stimulation was supplied by subcutaneous implantation of 10 percent stilbestrol-cholesterol pellets weighing 2.5 to 3.0 mg.

In the first experiment four groups of 1-to-3-months-old mice were used. The first group received the agent, estrogenic stimulation and the carcinogen; the second received the agent and estrogenic stimulation; the third, estrogenic stimulation and the carcinogen; and the fourth, the carcinogen alone. Each mouse was given applications of a 0.5 percent solution of the carcinogen twice a week for a total of 28 paintings.

Most of those receiving the agent and stilbestrol developed mammary tumors; none developed leukemia. Most of the painted mice developed leukemia (localized mediastinal tumors and general lymphomatosis) and skin tumors (papillomas and carcinomas). Estrogenic stimulation did not influence the occurrence of mammary tumors in carcinogen-painted mice.

In the second experiment two groups of 6-to-9-months-old mice were employed. One group was given estrogenic stimulation and the carcinogen; the other was given the carcinogen alone. Each animal received applications twice weekly of a 0.5 percent solution of the carcinogen for a total of 37 paintings.

Both groups showed high incidences (65 and 74 percent) of mammary tumors and high incidences of skin tumors, but low incidences of leukemia. Estrogenic stimulation did not increase the incidence of mammary tumors.

The third experiment included two groups of 2-to-5-months-old mice. One received estrogenic stimulation and the carcinogen; the other received the carcinogen alone. Each animal was given weekly applications of a 0.25 percent solution of the carcinogen for a total of 21 paintings.

Both groups showed medium incidences of mammary tumors (40 and 47 percent) and skin tumors (55 and 43 percent) but low incidences of leukemia (1 case in each group). Estrogenic stimulation again did not increase the incidence of mammary tumors.

Three groups of 1-to-5-months-old mice were used in the fourth experiment; none received estrogenic stimulation. One group was given the mammary tumor-agent and carcinogen; one the agent alone; and one the carcinogen alone. The painted mice each received a weekly application of a 0.25 percent solution of the carcinogen for a total of 14 paintings.

Both groups receiving the agent developed high incidences of mammary tumors (81 and 88 percent), whereas the group receiving the carcinogen alone showed an incidence of 31 percent. The group receiving the carcinogen alone showed an incidence of 26 percent skin tumors and the group receiving the agent and carcinogen showed an incidence of 3 percent

skin tumors. Both painted groups showed low incidences of leukemia (1 case in each group).

Throughout the experiments all the mice receiving the agent alone developed the typical acinous type of mammary gland tumor or adenocarcinoma usually encountered in high-mammary-tumor strains of mice carrying the agent, whereas the mice receiving the carcinogen alone, or together with stilbestrol, developed a variety of types of mammary gland tumors.

According to the experimental procedures described herein the following conclusions may be made.

1. Mammary gland tumors were induced in the agent-free females of this colony of strain dba subline 212 mice by percutaneous applications of methylcholanthrene dissolved in benzene. Estrogenic stimulation did not influence the occurrence of the induced mammary tumors.

2. The carcinogen also induced leukemias and skin tumors. Mice which were from 1 to 5 months of age when applications of the carcinogen began, showed a definite dose-response relationship in respect to the induction of leukemia and mammary tumor. The largest dose induced more leukemias than mammary tumors, but smaller doses induced many more mammary tumors than leukemias.

The medium dose elicited approximately the same number of mammary and skin tumors and the smallest dose more mammary tumors than skin tumors but the dose-response relationship in the induction of these tumors was not so pronounced as that shown by leukemias and mammary tumors.

3. Mice which were from 6 to 9 months old when painted for the first time developed fewer induced leukemias and more mammary tumors than did the younger animals.

4. Mammary tumors induced by the carcinogen and mice-bearing carcinogen-induced tumors did not contain the mammary tumor-agent.

5. Mammary tumors produced by the agent alone showed a regularly recurring histologic pattern whereas those induced by the carcinogen alone showed a variety of histologic types.

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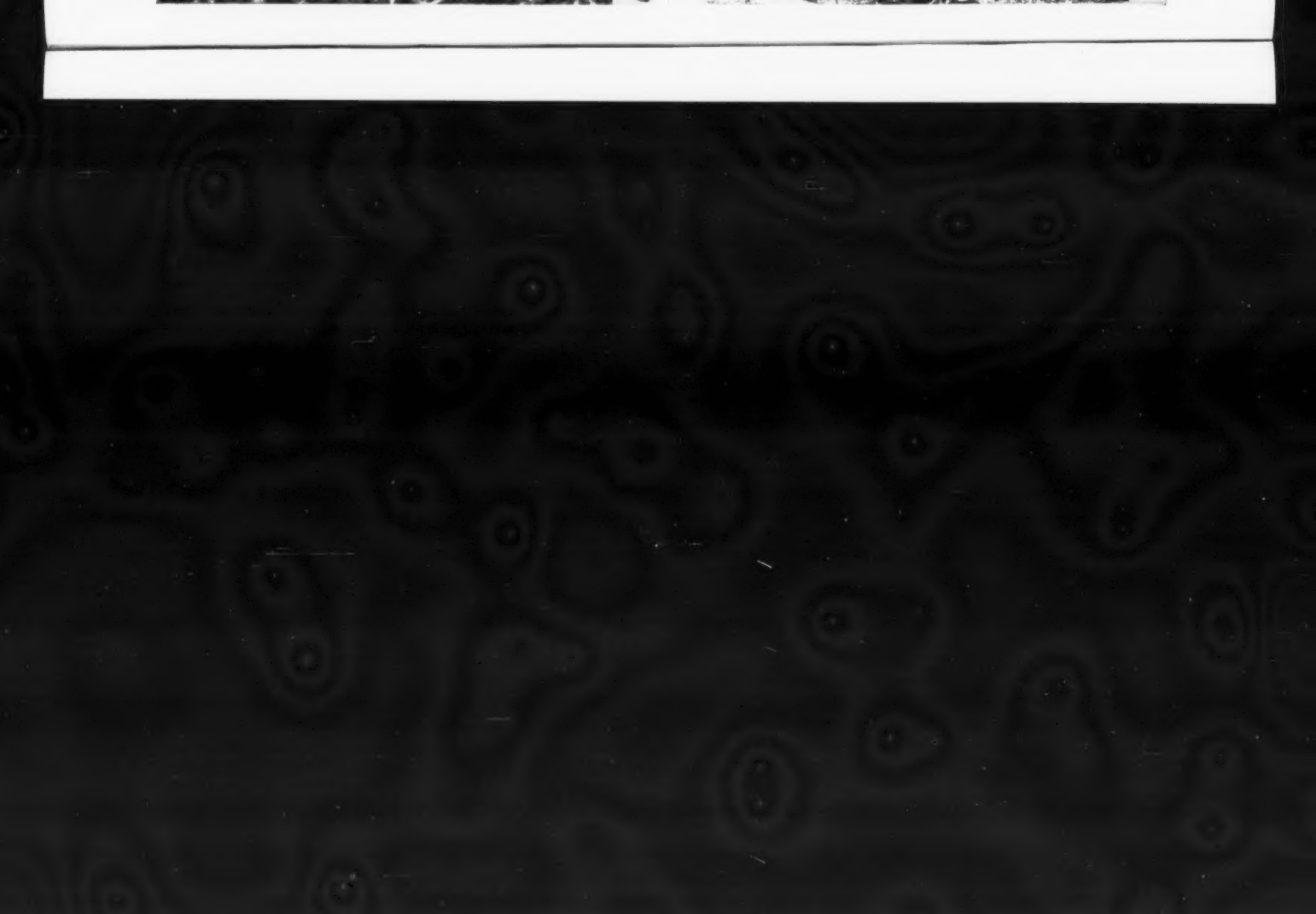
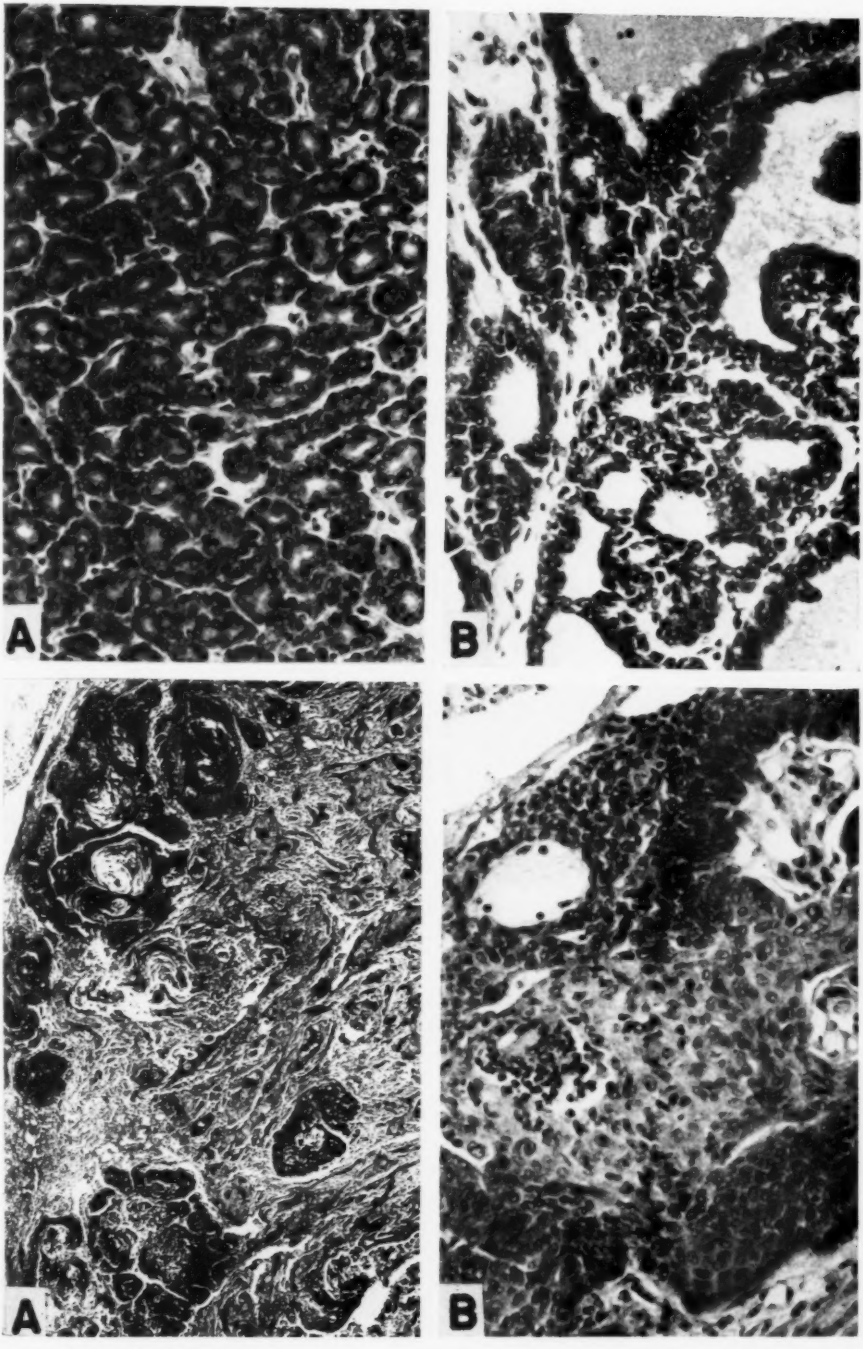
PLATE 135

FIGURE 1 (upper).—Adenocarcinomas, the typical or characteristic mammary tumors. *A.* Tumor reproducing structure of mammary acini; a basic acinar type. Dba mouse 11 months old. Experiment 1, tumor agent and stilbestrol. Hematoxylin and eosin. × 265. *B.* Cystic modification, but with acinar structure still apparent. Dba mouse 8 months old. Experiment 4, tumor agent only. Hematoxylin and eosin. × 265.

FIGURE 2 (lower).—Adenoacanthomas, or keratinized mammary tumors. *A.* Low-power view to show extent of acanthomatous area. Darker nodules of glandular tumor tissue are also seen. Dba mouse 11 months old. Experiment 3, stilbestrol and methylcholanthrene. Hematoxylin and eosin. × 36. *B.* High-power view of an adenoacanthoma, showing central keratinization in a nodule of glandular tumor tissue. Dba mouse 12 months old. Experiment 2, methylcholanthrene only. Hematoxylin and eosin. × 265.

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B

PLATE 136

FIGURE 3.—Adenoacanthomas, "molluscoid" form. *A*. Peripheral acinar tissue, with keratinized duct-like radiations toward center, suggesting a reproduction of entire mammary gland. Dba mouse 12 months old. Experiment 2, methylcholanthrene only. Hematoxylin and eosin. × 36. *B*. Tumor following similar pattern to *A*, with central keratinization. Dba mouse 11 months old. Experiment 3, stilbestrol and methylcholanthrene. Hematoxylin and eosin. × 36.

A

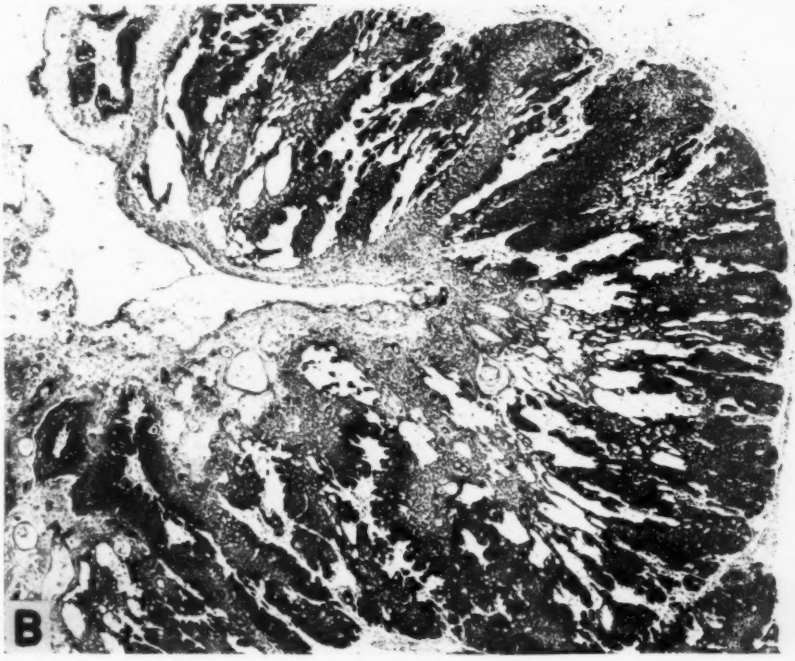

B

PLATE 137

FIGURE 4.—Carcinosarcomas. *A*. Merging of neoplastic cuboidal epithelial cells into elongate, spindle cells. Both types appear neoplastic. Dba mouse 14 months old. Experiment 3, stilbestrol and methylcholanthrene. Hematoxylin and eosin. × 265. *B*. Neoplastic glandular and spindle-cell elements appearing together. Note mitotic figures in both types of tissue. Dba mouse 7 months old. Experiment 1, methylcholanthrene only. Hematoxylin and eosin. ×265. *C*. Carcinosarcoma on left conjoined with a typical, solid adenocarcinoma on right. Dba mouse 13 months old. Experiment 2, methylcholanthrene only. Hematoxylin and eosin. × 265.

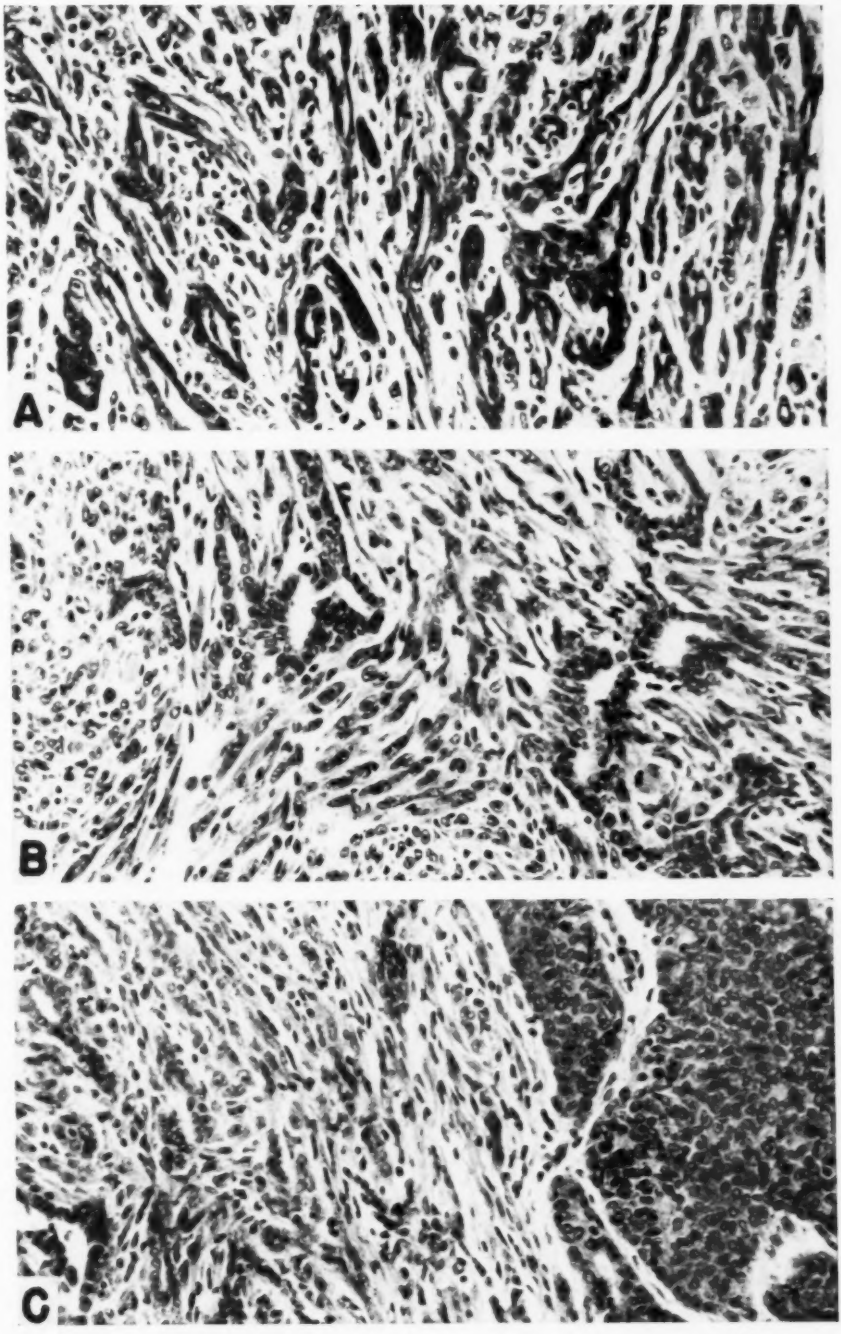
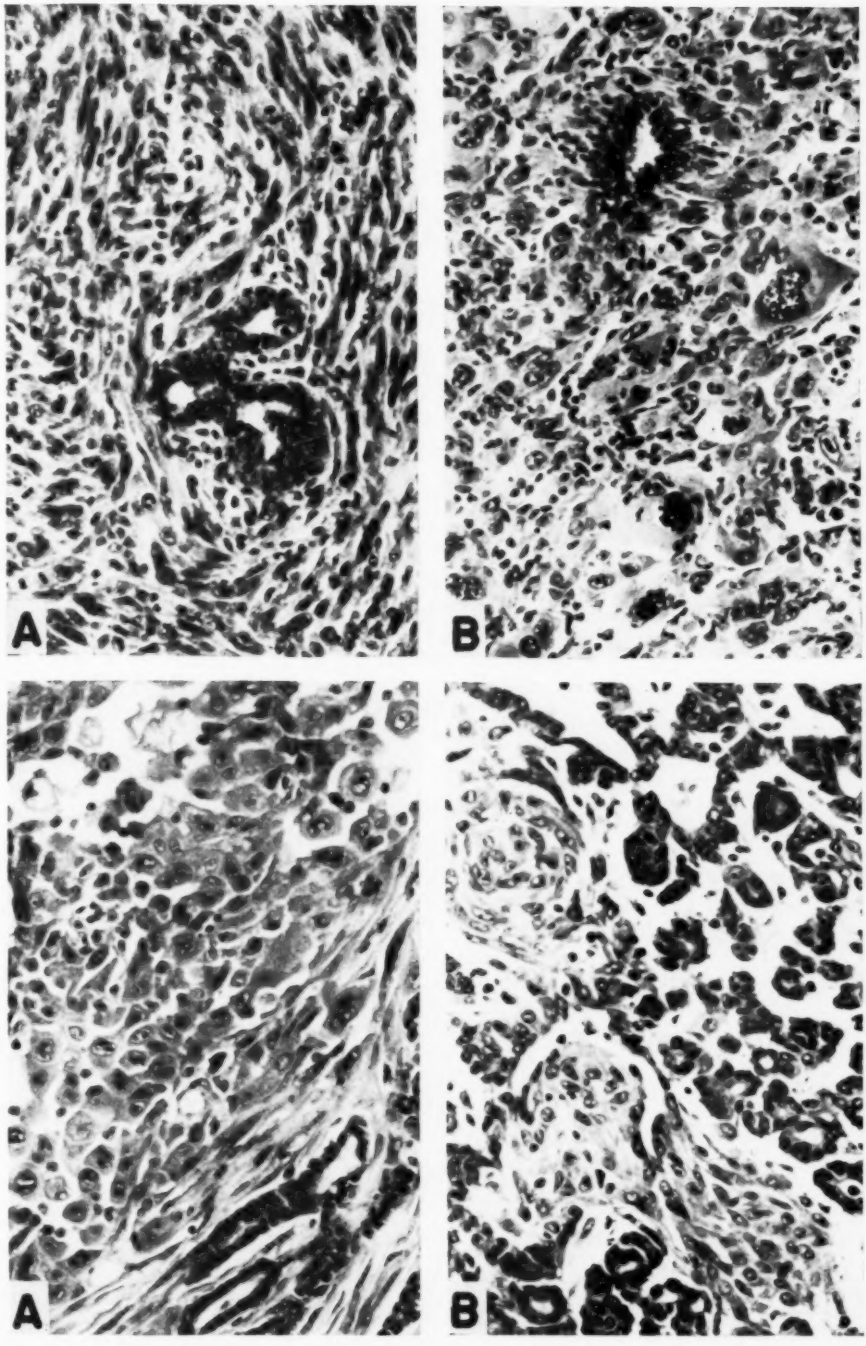

A
B
C

PLATE 138

FIGURE 5 (upper).—Sarcomas. *A.* Sarcoma with many mitotic figures is seen surrounding apparently normal mammary ducts. Dba mouse 14 months old. Experiment 4, methylcholanthrene only. Hematoxylin and eosin. × 265. *B.* Sarcoma with tumor giant cells surrounding mammary ducts. Tumors with such giant cells are frequently induced by methylcholanthrene. Dba mouse 18 months old. Experiment 4, methylcholanthrene only. Hematoxylin and eosin. × 265.

FIGURE 6 (lower).—Miscellaneous tumors. *A.* Tumor with areas composed of detached cells. Dba mouse 12 months old. Experiment 2, stilbestrol and methylcholanthrene. Hematoxylin and eosin. × 265. *B.* Tumor which does not fit any of usual categories for mammary tumors. Areas in which mammary acini are reproduced are interspersed with lightly-stained, whorl-like collections of elongated cells which may represent myoepithelial elements. Dba mouse 9 months old. Experiment 3, methylcholanthrene only. Hematoxylin and eosin. × 265.


A
B
A
B

INDUCTION OF HEMANGIO-ENDOTHELIOMAS AND SARCOMAS IN MICE WITH *O*-AMINOAZOTOLUENE

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During the past few years a series of experiments was performed in this laboratory to ascertain whether different carcinogens would reveal synergistic activity in the production of hepatomas in inbred mice. The studies failed to reveal conclusive evidence of synergism because the members of inbred strains did not respond uniformly to small doses of the carcinogens. Even litter mates showed wide variations in their degrees of susceptibility to the development of induced hepatomas.

O-aminoazotoluene was one of the carcinogens used in these investigations and received more attention than any other because it induced hepatomas, pulmonary tumors and hemangio-endotheliomas at sites remote from the site of administration (*1*) and, when injected subcutaneously, it evoked sarcomas at the site of injection (*2*). Subcutaneous administration of a 10-mg. dose to strain A female mice induced hepatomas and pulmonary tumors, and 20-, 30-, 40-, 50-, and 60-mg. doses induced hepatomas, pulmonary tumors, and hemangio-endotheliomas (*3*). When the compound was added to the diet and fed to strain C mice it induced hepatomas, pulmonary tumors, and hemangio-endotheliomas (*4*).

Various strains of mice, especially strain C, exhibited a striking sex difference in susceptibility to induced hepatomas; females were more susceptible than males (*1*, *2*, *4*). Gonadectomy and administration of testosterone propionate had a significant influence upon this sex difference in susceptibility to induced hepatomas (*5*). Castrate males were more susceptible than intact males; administration of testosterone propionate to castrate males and castrate females increased their resistance.

The primary objective in all these experiments was to determine the effect of various experimental procedures upon the occurrence of induced hepatic lesions and hepatomas; observations on the appearance of pulmonary tumors, hemangio-endotheliomas, and sarcomas were incidental. In a recent paper (*5*) some consideration was given to the appearance of hemangio-endotheliomas in the experimental animals, and it was suggested that in strain C mice there was a similarity between the production

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of *o*-aminoazotoluene-induced-hemangio-endotheliomas and hepatomas in respect to sex variation in susceptibility.

The purpose of this report is to present briefly a summary of the production of hemangio-endotheliomas and sarcomas by *o*-aminoazotoluene in experiments designed primarily to study the production of hepatomas and to report the results of one experiment which was performed to ascertain the sex susceptibility of strain C mice to induced hemangio-endotheliomas. Included also is a brief summary of a series of studies on the toxicity of *o*-aminoazotoluene for the mouse.

MATERIALS AND METHODS

All mice used in these experiments, with the exception of some strain A animals, were raised in this laboratory. The strain A mice which were not derived from colonies maintained at the National Cancer Institute were obtained from the Roscoe B. Jackson Memorial Laboratory. There were no significant differences in the responses of strain A mice from these sources. All mice were 2 to 3 months of age when the experiments were started, and with few exceptions (*1*) they were kept as virgins throughout the experiments.

With the exception of those that ingested the compound in the diet (*4*), all were fed Purina dog chow or laboratory chow. The diet was never changed during the course of an experiment, and tap water was available at all times. The mice were housed in wooden boxes, 28 cm. long, 18 cm. wide, and 15 cm. deep, with 8 to a box.

Two lots of *o*-aminoazotoluene were used. One was obtained from the Eastman Kodak Co. and was used without further purification. This lot was used in the majority of experiments already published and in most of the additional experiments reported herein. It was procured during 1937 and has been the source of unpurified compound for every experiment performed in this laboratory. The other lot was purified (m. p. 102.5°–103° C.) by Dr. J. L. Hartwell of the National Cancer Institute. It was used in only a few of the previously published experiments (*1*, *2*, *4*) and in some of the experiments on toxicity reported herein. The lots gave similar results in all experiments where both were used; hence, the results obtained in these investigations were not due to an impurity in the commercial preparation of the compound. A single oral or subcutaneous administration of only 4 mg. of the commercial preparation dissolved in olive oil induced hepatomas in strain A female mice (*6*).

The compound was injected into mice in crystalline form or in solution. When administered as crystals, it was moistened with glycerol (6 drops from a 1-cc. pipette to each 200 mg.). Crystalline material was not used for the administration of small amounts or when accurate dosage was essential. Unless stated otherwise all solutions were made in olive oil of U. S. P. quality by heating gently so that the flask was not too warm to hold in the palm of the hand. Fresh solutions were prepared for each injection.

Unless stated otherwise, all subcutaneous injections were made into the

right axillary region. Crystals moistened with glycerol were injected by means of a 12-gauge trocar with a snugly fitting plunger. Solutions were injected by means of a 1-cc. syringe and a 20-gauge needle. The point of entrance of the needle through the skin was sealed with collodion to prevent escape of the oily solution. Oral administration into the stomach was carried out by using a 1-cc. syringe and a blunt, curved, 20-gauge needle, according to the technique described by Morris and Thompson (*7*).

Necropsies were performed on all mice, and all tissues were fixed in Tellyesniczky's fluid (2 parts formol, 20 parts 70 percent alcohol, and 1 part acetic acid). Sections were prepared from virtually all tumors, and the diagnosis was based upon histologic findings. Gross and microscopic hepatic changes are not emphasized in this paper, but these have been described previously (*1*, *5*, *8*). Induced pulmonary tumors have also been described (*1*). References to the histologic appearance of induced hemangio-endotheliomas will be given later in this paper.

EXPERIMENTAL PROCEDURES AND RESULTS

Toxicity Studies

A number of experiments were performed to ascertain the maximum dose tolerated when given at a single injection and the ability of the mice to survive repeated injections of measured amounts of the compound. Mice of strains C and A were studied most intensively because strain C revealed a striking sex difference in susceptibility to induced hepatomas and strain A was considered to be the most susceptible to induced hepatomas.

Details of these experiments will not be presented. All were carried out with olive-oil solutions, and all oral injections were given to mice that had been deprived of food 15 hours previously. Results with strain C mice are summarized as follows:

(1) Following 1 subcutaneous injection of 60 mg. in 0.5 cc., all females and males died within 2 days.

(2) Following 1 subcutaneous injection of 40 mg. in 0.5 cc., all females and males died within 1 week. Following 1 oral injection of the same solution, all females and males died within 2 days.

(3) Following subcutaneous injection of 30 mg. in 0.5 cc., 3 of every 4 females and males died within 1 month. Following oral injection of the same solution, all females and males died within 2 days.

(4) Following subcutaneous injection of 20 mg. in 0.5 cc., all females and males survived. Following 1 oral injection of the same solution all females and males died within 1 week.

(5) Following 1 subcutaneous or oral injection of 10 mg. in 0.5 cc., all females and males survived.

These findings following a single administration, showed that the compound was far more toxic for strain C mice when injected orally than when injected subcutaneously. The results of repeated administrations were as follows:

(1) Following 3 subcutaneous injections of 10 mg. in 0.25 cc. each, at

intervals of 1 week, all males lived. Following oral injections of the same solutions, 1 of every 3 males died.

(2) Following 4 subcutaneous injections of 10 mg. in 0.25 cc., 0.5 cc., or 1.0 cc. each, at intervals of 4 weeks, all females lived. Following oral injections of the same solutions 1 of every 4 females died.

These findings also indicate that the compound was more toxic when administered by the oral route. Survival of the test animals does not imply that the dose was not toxic because weight records were kept of strain C females which received 10 mg. of the crystalline compound at intervals of 1 month. After each injection the average weight of groups of young mice remained stationary for several days, and full grown mice lost an average of 1 gm. in body weight within 14 days and required 7 more days to regain the lost weight.

Another interesting feature of the toxicity studies with strain C mice was the similarity of response in males and females despite the fact that females were far more susceptible to the induction of hepatic lesions and hepatomas than were the males. Every female survivor of the toxicity tests that lived 6 months or longer developed hepatic lesions, and most of them also developed hepatomas but none of the male survivors developed a gross or microscopic hepatic lesion.

Tests performed to ascertain the toxicity of the compound for strain A females are presented at this time in order to compare them with the results obtained in strain C. The results attending the use of strain A females are summarized below:

(1) Following 1 oral injection of 20 mg. in 0.5 cc., all died within 3 days.

(2) Following 1 subcutaneous or oral injection of 10 mg. in 0.25 cc., all lived.

(3) Following 2 oral injections of 10 mg. in 0.25 cc. each, at an interval of 1 week, all lived. Following 3 such oral injections, 3 of every 4 died within 1 week of the last injection.

(4) Following 5 subcutaneous or oral injections of 10 mg. in 0.25 cc. each, at intervals of 4 weeks, all lived.

These findings with strain A females indicate that, although the A females were considerably more susceptible to the hepatoma-inducing power of the compound than were the C females, there were no pronounced differences in their susceptibilities to its toxic activity.

Induction of Hemangio-endotheliomas and Sarcomas

The occurrence of hemangio-endotheliomas and sarcomas in mice following administration of the compound was mentioned in previous reports (*1–5*), and a summary of the occurrence of hemangio-endotheliomas in strain C mice was presented in a recent publication (*5*).

A complete summary of the induction of these tumors in various strains of mice is presented here. For the sake of brevity, detailed descriptions of these experiments are not included, and the results are

summarized in table 1. The findings of the first 5 experiments of the table have been published, whereas those of the last 7 are presented for the first time. It must be remembered that none of these experiments was designed primarily to ascertain the induction of hemangio-endotheliomas or sarcomas. The usual procedure was to kill some mice, 3, 6, 9, 12, and 15 months after the first administration of *o*-aminoazotoluene to determine the presence of hepatic lesions. Hence, all the mice of every experiment are not included in the table; only those groups of which one or more developed hemangio-endotheliomas or sarcomas are presented.

Experiment 145 was performed to ascertain the influence of the solvent upon the induction of hepatomas in strain C females, and the entire experiment is included in the table. The solvents did not exert any significant influence upon the ability of the carcinogen to induce hepatomas but the crystalline compound elicited the greater number of hemangio-endotheliomas whereas the olive oil solution was the only solution to produce sarcomas.

Experiment 156 included 100 C females each of which received 30 mg. of the compound, but only 6 developed hemangio-endotheliomas. The fact that the majority of the mice developed hepatic lesions and hepatomas showed that they were more susceptible to induced hepatomas than induced hemangio-endotheliomas.

Experiments 157 and 163 were tests for toxicity in strain C mice. The groups included in table 1 show that sarcomas were induced by one subcutaneous injection of 20 mg. or 30 mg. of the compound in olive oil, by 2 injections of 15 mg. each and by 8 injections of 3.75 mg. each.

Experiments 164 and 166 were also tests for toxicity in strain C females. The results of experiment 166 suggested that 4 injections of 10 mg. each in olive oil may be a satisfactory procedure for the induction of sarcomas at the injection site.

Experiment 174 was included to show that an olive-oil solution of the carcinogen induced sarcoma in a strain A female. The data in table 1 permit the following conclusions:

(1) Subcutaneous administration of the compound in crystalline form induced hemangio-endotheliomas at a variety of sites but did not induce sarcomas.

(2) Crystalline material elicited more hemangio-endotheliomas than did olive-oil solutions, but the latter induced all the sarcomas.

(3) When the crystalline material was injected into the right axillary region, most of the induced hemangio-endotheliomas arose in the interscapular fat, but when it was injected at the base of the tail most of these neoplasms arose in the lungs.

(4) When the compound was incorporated in the diet, most of the induced hemangio-endotheliomas arose in the lungs.

(5) The compound induced hemangio-endotheliomas in mice of strains C, A, C3H, and C57 black.

In addition to the hemangio-endotheliomas and sarcomas, *o*-aminoazotoluene also induced adenomatous pulmonary tumors. Mice of strain A were highly susceptible to this type of induced neoplasm, and next in order of susceptibility were animals of strain C.

TABLE 1.—*Summary of occurrence of hemangio-endotheliomas and sarcomas in inbred mice following administration of o-aminoazotoluene*

Reference or experiment No.	Site of subcutaneous administration	Condition of compound	Solvent	Dose each injection	Number of injections	Total dose	Strain	Sex	Number of mice	Distribution of hemangio-endotheliomas according to site of origin										Total	Mice developing sarcoma at injection site
										Interscapular fat	Lung	Peritoneal fat	Subcutaneous tissues	Ovary	Adrenal gland	Muscle	Spleen	Liver	Pleural cavity		
				Mg.		*Mg.*				*Number*	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*
(1)	Right axilla	Crystalline [1]		10	10	100	A	F	11						1					1	
(1)	do	do		10	10	100	A	M	11				1							1	
(1)	do	do		10	10	100	C3H	M	23				1							1	
(1)	do	do		10	10	100	C	F	12	8	1	1	2	1	1					14	
(1)	do	do		10	10	100	C	M	12	2			2							4	
(1)	do	do		10	10	100	C57B1	F	15	4										4	
(1)	do	do		10	10	100	C57B1	M	34	8										8	
(2)	do	Solution	Olive oil	5	9	45	C57B1	F	21	1								1		2	3
(2)	do	do	do	5	9	45	C57B1	M	20	2										2	
(2)	do	do	do	5	9	45	C3H	F	8												1
(2)	do	do	do	5	9	45	C	F	20	3				1						4	1
(2)	do	do	do	5	9	45	C	M	20	1			1							2	
(3)	do	Crystalline		10	2	20	A	F	20			1								1	
(3)	do	do		10	3	30	A	F	20	1										1	
(3)	do	do		10	4	40	A	F	20	1		1		1						3	
(3)	do	do		10	5	50	A	F	20	2		2		1						5	
(3)	do	do		10	6	60	A	F	20	3		2	1							6	
(4)		In diet				100-165	C	F	31	1	16	1								18	
(4)		do				130-220	C	M	29		4									4	
(5)	Base of tail	Crystalline		10	7	70	C	[2] F	45	9	23		2			1				35	
(5)	do	do		10	7	70	C	[2] M	49	6	15									21	
(5)	Right axilla	do		10	4	40	C	[2] F	125	7		2	1				1			11	
(5)	do	do		10	4	40	C	[2] M	129	1		1	4				1	1		8	
145	do	Solution	Olive oil	5	8	40	C	F	20					1						1	4
145	do	do	Sesame oil	5	8	40	C	F	20	3				1						4	
145	do	do	Tricaprylin	5	8	40	C	F	20	1		2								3	
145	do	Crystalline		5	8	40	C	F	20	3		3	1	3					2	12	
156	do	do		10	3	30	C	F	100	1		1		4						6	

157	do	Solution	Olive oil	20	1	20	C	M	5					2							1
157	do	do	do	15	2	30	C	F	36											2	2
157	do	do	do	3.75	8	30	C	F	18												1
157	do	do	do	30	1	30	C	F	17												1
163	do	do	do	20	1	20	C	M	5												1
164	do	do	do	10	2	20	C	F	20												1
166	do	do	do	10	4	40	C	F	39												5
174	do	do	do	10	2	20	A	F	8												1

[1] 200 mg. of compound moistened with 6 drops of glycerol.
[2] Includes intact and castrate animals.

In a previous paper (*5*) an analysis of the results obtained following administration of *o*-aminoazotoluene to strain C mice indicated that females were more susceptible than males to induced hemangio-endotheliomas. It was also shown that administration of the carcinogen subcutaneously at the base of the tail induced many hemangio-endotheliomas in the lungs. An experiment was performed to ascertain (1) whether strain C mice showed a sex difference in susceptibility to induced hemangio-endotheliomas and (2) whether hemangio-endotheliomas arose in the lungs when the carcinogen was applied at another site.

Twenty litters of strain C mice 2 to 5 months of age were used. They were maintained as virgins, kept under similar environmental conditions, and fed a diet of Purina laboratory chow. The injections were arranged so that male and female litter mates received the carcinogen in the same injection sites.

Crystalline *o*-aminoazotoluene procured from the Eastman Kodak Co. was used. It was moistened with glycerol and injected into 4 sites. These were: subcutaneously in the interscapular region; subcutaneously at the base of the tail; subcutaneously in the mid-dorsal region; and intraperitoneally. Each mouse received approximately 10 mg. at each injection. A total of 7 injections was given; the first 4 at monthly intervals, followed by an interval of 3 months, and then the last 3 injections at monthly intervals. Thus each mouse received a total of 70 mg. of the crystalline compound over a period of 8 months.

This was the only experiment of the entire series that was performed primarily to ascertain the induction of hemangio-endotheliomas, and the mice, instead of being killed at regular intervals after injection, were kept as long as possible. Some were killed because of the presence of large tumors in the interscapular region and the others when they were obviously ill. Some died suddenly with massive hemorrhage into the pleural cavity. These deaths were always associated with the presence of a large hemangio-endothelioma in the lungs. The experiment was terminated 15 months after the first injection, when only male mice were surviving.

Necropsies were performed on all mice. A piece of liver and all gross hemangio-endotheliomas were prepared for histologic study. The latter were easily distinguished by their red color. The lungs were examined for the presence and number of macroscopic pulmonary tumors but precise records could not be made because at times a large hemangio-endothelioma had filled an entire lobe.

All tumors were diagnosed histologically. Around the larger hemangio-endotheliomas there was usually a wide zone of dilated vessels and hemorrhage, and if the section happened to pass only through this peripheral zone, the neoplastic cells were not seen. Such sections were diagnosed as hemangio-endotheliomas only when the gross diagnosis revealed a large hemorrhagic mass, which, through experience, could safely be called a neoplasm of this type.

The occurrence of hemangio-endotheliomas is summarized in table 2. The over-all results showed a decided sex difference in susceptibility to this neoplasm for, with the exception of those mice that had received the compound in the interscapular region, the females developed many more tumors than did the males.

TABLE 2.—*Occurrence of hemangio-endotheliomas in strain C mice following administration of 70 mg. of crystalline o-aminoazotoluene*

Number of mice autopsied
Number with hemangio-endotheliomas

Route of administration	Mice	Sex	Time after first injection in months								Total
			8	9	10	11	12	13	14	15	
	Number										
Subcutaneous in interscapular region	16	F	1/0	1/0	2/1	5/3	5/2	1/1	1/0	----	16/7
	13	M	1/0	2/0	----	----	----	2/2	3/1	5/1	13/4
Subcutaneous at base of tail	18	F	----	1/1	2/2	4/3	6/4	3/2	2/0	----	18/12
	12	M	1/0	----	----	----	1/0	----	2/1	8/0	12/1
Subcutaneous in mid-dorsal region	16	F	----	1/1	3/3	2/2	5/3	3/3	2/2	----	16/14
	13	M	1/0	1/0	----	----	----	2/1	1/1	8/0	13/2
Intraperitoneal	16	F	----	3/0	1/1	3/2	1/0	2/1	6/1	----	16/5
	11	M	----	1/0	----	----	----	----	4/0	6/0	11/0

Data in table 2 also show that the carcinogen induced the most hemangio-endotheliomas in strain C females when injected into the mid-dorsal region or at the base of the tail and the least when injected interscapularly or intraperitoneally. An explanation for this difference is found in table 3, which summarizes the sites of origin of the tumors. When injected at the base of the tail and mid-dorsally, the compound elicited a considerable number of hemangio-endotheliomas in the lungs of the females.

TABLE 3.—*Summary of sites of origin of hemangio-endotheliomas induced in strain C mice with o-aminoazotoluene*

Route of administration	Mice	Sex	Distribution of hemangio-endotheliomas according to site of origin							Total
			Interscapular fat	Lung	Peritoneal fat	Subcutaneous tissues	Ovary	Muscle	Spleen	
	Number									
Subcutaneous in interscapular region	16	F	6	1	----	----	----	----	----	7
	13	M	1	----	2	1	----	----	----	4
Subcutaneous at base of tail	18	F	2	8	----	1	1	----	----	12
	12	M	1	----	----	----	----	----	----	1
Subcutaneous in mid-dorsal region	16	F	7	8	1	1	1	1	1	20
	13	M	2	----	----	----	----	----	----	2
Intraperitoneal	16	F	1	3	----	1	1	----	----	6
	11	M	----	----	----	----	----	----	----	0

The reasons why the carcinogen evoked more hemangio-endotheliomas of the lung when administered at certain sites is not known, but these results together with those of previous experiments (*4*, *5*) indicate clearly that the route and site of administration of *o*-aminoazotoluene are important factors in determining where it will produce this type of neoplasm.

Table 3 also shows that none of the males developed hemangio-endotheliomas of the lungs. In an earlier investigation (*5*), 7 intact and 42 castrate strain C males were given the compound at the base of the tail, and of 17 castrates that developed hemangio-endotheliomas, 15 had lung tumors of this type. None of the intact males developed a hemangio-endothelioma of the lungs. This finding together with the results of the present study indicates that gonadectomy of strain C males increased their susceptibility to induced hemangio-endotheliomas of the lungs.

As in previous experiments all the females developed hepatic lesions, and, with few exceptions, they also developed hepatomas, whereas only a few of the males had hepatic lesions; none developed a hepatoma. For reasons stated above, precise records of the occurrence of adenomatous pulmonary tumors could not be obtained, but the available data indicated that there was no significant sex difference in susceptibility to this neoplasm. Thus, the results of the experiment showed that strain C females were more susceptible than males to *o*-aminoazotoluene-induced hepatomas and hemangio-endotheliomas, but both sexes were equally susceptible to induced pulmonary tumors. None of the mice developed a sarcoma.

DISCUSSION

Spontaneous tumors of the endothelium of blood vessels are uncommon in mice. In 1930 Foulds (*9*) reviewed the literature and found descriptions of 7 such tumors of which 2 arose in the subcutaneous tissues and 1 each in the groin, scrotum, omentum, mammary gland, and abdominal cavity. He observed 9 additional tumors of which 8 arose in the subcutaneous tissues and 1 in the lip. The tumors included in Foulds' report were found in mice of unknown ancestry and before the establishment of inbred strains with known incidences of spontaneous tumors. Thus far there are no reports of any inbred strain in which hemangiomas occur frequently. Cloudman (*10*) in his chapter on spontaneous neoplasms in mice in the Biology of the Laboratory Mouse described the types of spontaneous tumors most commonly encountered in the inbred strains maintained at the Roscoe B. Jackson Memorial Laboratory. He mentions that hemangiomas in the mammary-gland region were found in the C57 black strain and that cavernous hemangiomas arose in the digestive systems of the C57 black and dba strains as well as in their hybrids, but did not give the incidence for either strain or hybrids. He also stated, without further information, that hemangiomas and hemangio-endotheliomas occurred in the dermis, in subcutaneous and body wall tissue, in the liver, and in the ovaries of inbred mice.

The exact incidence of spontaneous hemangio-endotheliomas in the inbred-mice strains maintained in this laboratory (strains C3H, C, C57

black, I, Y, R3, and dba) is not known, but none is higher than 5.0 percent. A spontaneous malignant hemangio-endothelioma of the liver in a strain C3H male has been described (*11*).

Different kinds of carcinogenic agents have been implicated in the production of hemangiomatous lesions in mice. The majority of such reports deal with the use of carcinogenic hydrocarbons. Furth and Furth (*12*) found 2 cases of hemangioma, 1 of the liver, and 1 of the ovary, in mice that had received a splenic injection of benzpyrene dissolved in lard, and hemangiomas of the spleen and a lymph node in a mouse that had been given intravenous injections of a colloidal suspension of dibenzanthracene. Shear and Ilfeld (*13*) implanted dibenzanthracene-cholesterol pellets into the interscapular region of strain dba mice and found 2 hemangiomas "at or near the pellet" and 1 hemangiomatous lesion "at a site where the pellet might possibly have been." They concluded that their results in respect to hemangiomas "raises the question as to whether they were induced or spontaneous."

Other investigators reported clear-cut evidence of the production of hemangiomas in mice by carcinogenic hydrocarbons. Steiner (*14*) inserted methylcholanthrene-cholesterol pellets into the salivary glands and induced 24 salivary gland tumors of which 3 were cavernous hemangiosarcomas. Hartwell and Stewart (*15*) applied 5,9,10-trimethyl-1,2-benzanthracene to the skin of 102 mice and found that 4 (1 strain dba and 3 F_1 hybrids from strains dba and C57 black) developed hemangio-endotheliomas in association with squamous-cell carcinomas. White and Stewart (*16*) incorporated methylcholanthrene in a diet that was fed to mice for a period of 6 months: the mice ingested an average of 1 mg. of the carcinogen per day per mouse. Fifty strain C3H and 32 strain C mice which were 2 months of age and equally divided as to sex were used. Eleven strain C3H and 21 strain C mice developed hemangio-endotheliomas. The tumors were equally frequent in males and females. With the exception of one tumor, all arose in the small intestines, mesentery, pancreas, or mesenteric lymph node. One mouse had metastasis to the liver and another to the lung and retroperitoneal lymph node. This paper contains a detailed description of the histologic appearance of the induced hemangiomas. Lorenz and Stewart (*17*) reported the results of oral administration of aqueous olive-oil emulsions of either methylcholanthrene or dibenzanthracene in place of drinking water. The dibenzanthracene emulsion was given to males of strains C3H and C57 black and males and females of strains dba and A as well as backcross animals derived from strain A. Hemangio-endotheliomas of the mesentery or peritoneum occurred in 1 of 20 C3H males, 4 of 10 dba males, 2 of 10 dba females, and 1 A backcross male.

Selbie (*18*) as well as Andervont and Shimkin (*19*) found hemangiomas in mice at the site of subcutaneous administration of 0.2 cc. colloidal thorium dioxide. Grady, Blum, and Kirby-Smith (*20*) described the types of tumors induced in strain A mice which were subjected to a wide range of dosages and intensities of ultraviolet radiation. Tumors of the

ears of 605 mice were examined, of which 5 were hemangio-endotheliomas. They also found tumors in the eyes of about 5 percent of the mice. Such tumors were either sarcomas or hemangio-endotheliomas most of which arose in the substantia propria of the cornea.

The foregoing investigators have shown that hemangiomatous tumors can be induced in mice by a variety of carcinogens, and the tumors can be evoked in various inbred strains and their hybrids. This paper records the occurrence of hemangio-endotheliomas in certain inbred strains of mice following administration of *o*-aminoazotoluene but does not establish which strains are most susceptible or resistant to the induced tumor. Strain C was considered to be relatively susceptible and has received considerable attention because the results of previous investigations had suggested, and those of an experiment described herein confirmed, the greater susceptibility of females. Furthermore, gonadectomy increased the susceptibility of males to induced hemangio-endotheliomas (*5*).

A sex difference in susceptibility to hemangiomas induced by other carcinogens has not been observed. The only reference in this respect is a statement by White and Stewart (*16*) that the mice in which hemangio-endotheliomas occurred following oral administration of methylcholanthrene were equally divided as to sex. Further work is needed to ascertain whether a sex factor is involved in the susceptibility of mice to hemangiomatous lesions induced by other carcinogens and whether other inbred mouse strains also exhibit a sex difference in susceptibility to *o*-aminoazotoluene-induced hemangiomas.

It is of interest to note that Watson and McCarthy (*21*) in their study of spontaneous hemangiomas in humans emphasized the higher incidence in females.

The results of the experiment presented herein establish crystalline *o*-aminoazotoluene as an effective carcinogen for eliciting hemangio-endotheliomas in mice and also again emphasizes the importance of the site of administration of the crystals in the production of pulmonary hemangio-endotheliomas. It induced many of these neoplasms in the lungs when injected subcutaneously in the mid-dorsal region and at the base of the tail and few when injected interscapularly.

The summary in table 3 shows that the induced pulmonary hemangio-endotheliomas arose in the females only. This again reveals the greater susceptibility of the females. In an earlier experiment (*4*) larger amounts of the carcinogen were administered to strain C mice in their diet, and 16 of 31 females and 4 of 29 males developed pulmonary hemangio-endotheliomas. This showed that the lungs of males of strain C are not completely resistant to these induced neoplasms.

During the course of the investigations summarized in this paper, a number of interscapular hemangio-endotheliomas were transplanted successfully into other mice of the strain in which they originated. They usually grew rapidly and developed blood-filled cysts. Serial transplantation was accomplished by using the usual trocar technique to im-

plant pieces of the cyst wall subcutaneously into mice of the same strain. All these tumor strains have been discontinued.

The histologic appearance of induced hemangio-endotheliomas has been described by pathologists (*1, 5, 16, 20*) of the National Cancer Institute.[2] The tumors are composed of atypical blood-vessel endothelium which forms both vascular channels and solid masses.

The production of subcutaneous sarcomas by *o*-aminoazotoluene requires little discussion since the occurrence of these tumors has been recorded from this laboratory (*2*) and elsewhere (*22, 23*). Thus far they have not appeared following injections of the crystalline compound. This implies that *o*-aminoazotoluene is more carcinogenic for subcutaneous tissues when in solution. The results of experiments 157 and 163 recorded here (table 1) extended earlier observations by showing that a single subcutaneous injection of an olive-oil solution induced sarcomas in strain C mice. Heretofore all such tumors had appeared after the mice had received multiple injections.

The fact that the induced sarcomas arose at the site of subcutaneous administration is of special interest for it shows that the carcinogenic activity of *o*-aminoazotoluene is not limited to tissues distant from the site of application. The compound is now known to induce hepatomas, pulmonary tumors, and sarcomas, as well as hemangio-endotheliomas, in a variety of sites.

SUMMARY

Experimental observations recorded herein provided information concerning the toxicity of *o*-aminoazotoluene for inbred mice and the ability of the compound to induce hemangio-endotheliomas and sarcomas in inbred mice.

Studies on the toxic action of the compound showed that in mice of strain C it was more toxic when administered orally than subcutaneously.

Male and female mice of strain C responded similarly to the toxic activity of the compound despite the fact that females were far more susceptible than males to its hepatoma-inducing ability.

There was no pronounced difference in the susceptibilities of strain A and C females to the toxic action of the compound despite the greater susceptibility of strain A females to its hepatoma-inducing power.

Studies on the carcinogenic action of the compound showed that it was capable of producing hemangio-endotheliomas and sarcomas in mice of strains C, A, C3H, and C57 black. The hemangio-endotheliomas arose at a variety of sites which were distant from the site of application, whereas the sarcomas always appeared at the site of subcutaneous administration.

Olive-oil solutions induced both hemangio-endotheliomas and sarcomas, but the crystalline compound induced hemangio-endotheliomas only.

Females of strain C were more susceptible than males to the induction of hemangio-endotheliomas.

[2] Those reported for the first time in this paper were examined by Dr. Thelma B. Dunn of the National Cancer Institute.

When the crystalline material was injected subcutaneously into the right axillary region of strain C females, most of the induced hemangio-endotheliomas arose in the interscapular fat, but when it was injected subcutaneously at the base of the tail or mid-dorsally, or when administered orally, the majority of these neoplasms occurred in the lungs.

These differences in the susceptibility of strain C mice to the hemangio-endothelioma-inducing properties of *o*-aminoazotoluene offer further opportunities for studies on the mechanism of tumor formation by this compound.

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EXTENSION OF CELLOPHANE SUBSTRATE PROCEDURE TO GROWTH OF *IN VITRO* CULTURES OF LARGE AREAS [1]

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INTRODUCTION

In a previous publication Evans and Earle (*1*) have described the use of perforated cellophane sheets as substrates for the growth of a number of cell types in tissue culture in Carrel flasks of 35 and 50 mm. diameter. In the largest cultures reported sheets of Strain L mouse sarcoma cells successfully covered perforated cellophane disks measuring up to about 45 mm. in diameter in less than 32 days. The culture fluid routinely used in this work consisted of 40 percent horse serum, 20 percent (1:1) chick embryo extract from 9-day-old embryos, and 40 percent Earle's saline.

Many potential applications of tissue culture procedure could be realized if it were practicable to grow cultures of larger size. The ease with which it was found possible to obtain cell sheets 45 mm. in diameter suggested that the use of a perforated cellophane sheet substrate might allow the practical growth of culture of far larger size.

The present paper reports a series of experiments in which the use of such perforated cellophane-sheet substrates has allowed the growth of cultures of satisfactory cell density and far larger area than we have formerly considered practicable by older methods.

MATERIALS AND METHODS

Two experiments will be described. In the first the culture vessels used were Carrel D-type flasks of approximately 80 mm. internal diameter and 28 mm. internal height. In the second Blake-type culture bottles of 1 liter capacity were used.

Perforated cellophane used as a substrate for all cultures was DuPont PT 62, thickness 450, with perforation designs 2B,[3] 2A and OC (*2*)

[1] Received for publication December 13, 1949.

[2] With the technical assistance of E. P. Wilson, E. M. Duchesne, and M. F. Edward.

[3] Designs of the Beckley Perforating Co., Garwood, N. J. Design OC of this cellophane is now available from the Laboratory of the Tissue Culture Commission, Sloan-Kettering Institute Building, 444 East 68th St., New York 21, N. Y. Design OC is now used as standard in the Tissue Culture Unit of the National Cancer Institute. This design has 324 holes, each of 0.023-inch diameter to the square inch, the holes being arranged in a diagonal pattern, 45° center. This cellophane had 13½ percent open area.

Since the perforation design did not appear to influence the results in these studies appreciably the exact design will not be specified for each experiment.

Cellophane sheets were cut to fit the floor of each type of flask: for the D–80 flask a circle 75 mm. in diameter, approximately 45 square centimeters in area after autoclaving, was used; for each Blake flask the cellophane strip 80 mm. wide (the full width of the perforated cellophane strip available), and approximately 200 mm. long was used. These sheets were cleaned in alcohol, ether, acetone, and distilled water as previously described (*2*). One sheet of correct size was inserted into each culture flask and autoclaved.

The culture fluid used in the whole series of studies was that previously described (*3*) and consisted of 40 percent horse serum, sterilized by filtration under pressure through a # .03 Selas filter, 20 percent of 1:1 chick embryo extract and 40 percent Earle's saline. This culture fluid was changed three times weekly.

Two strains of cells were used in this work. The first was a fibroblast strain grown from freshly explanted 9-day-embryo chick heart. The second was strain L mouse sarcoma cells (*3*, *4*, *5*). This strain has now been maintained in culture in this laboratory for more than 7 years. These cell strains were chosen because a great deal of work had already been done with them, so that their general behavior was well known.

Experiment 1: Four sets of cultures were explanted into D–80 flasks; the explants were 5 by 15 mm. strips cut from rapidly growing plasma cultures of strain L.

Set A. A control set, of 2 cultures.

The explant strips in this set were planted in a matrix made up of 3.3 ml. chicken plasma and 4.1 ml. of the fluid medium described above. After clotting, this was overlaid with 5.5 ml. of the fluid medium, which was changed three times weekly.

Set B included 2 cultures in which one strip of strain L cells was explanted under perforated cellophane which had been washed in 1 ml. of culture fluid. One ml. of culture fluid was used at each fluid change.

Set C included 3 cultures, in each of which one strip explant was planted under perforated cellophane which had been soaked with 5.5 ml. of fluid medium. Each culture was given 5.5 ml. of the same fluid medium at each fluid change.

Set D included 2 cultures, in each of which 5 strips of strain L cells were explanted under perforated cellophane. Culture fluid used was as in set C.

Set E included 2 cultures, one was planted with 2 ml. and the other with 3 ml. of a diffuse cell suspension prepared by scraping the cell sheet growing on the cellophane surface and glass floor of 2 and 3 Carrel D–3.5 cultures. Each D–3.5 culture used as a parent culture contained 1 ml. of fresh culture fluid during the scraping.

Experiment 2: Four sets of cultures were planted in 1-liter Blake bottles, using a cellophane substrate.

Set A included 2 bottles each seeded with chick fibroblasts scraped by

means of a platinum spatula from the cell sheet in one D–3.5 Carrel flask culture. One of these cultures was lost by infection.

Set B included 2 bottles, one of which was lost due to an infection. Cultures of this set were similar to those of A except each was seeded with the cells from two D–3.5 flask cultures.

Set C included 2 bottles and was similar to A except each bottle was seeded with cells from 4 D–3.5 flask cultures.

Set D was similar to A except that one bottle was seeded with cells from 5 D–3.5 flask cultures of strain L cells.

Each culture was started with an initial volume of 9 ml. of fluid culture medium. In the more densely cellular cultures, as growth of the culture appeared to justify, this volume was increased. For instance at 31 days, in cultures of set C the fluid was increased to 15 ml.; it was further increased to 30 ml. at 50 days. All cultures still living were closed at 79 days.

RESULTS

Experiment 1: Set A plasma control cultures showed only moderate growth at 6 days, but by 18 days, growth was abundant, the cells spreading out in a wide fringe from the explant. By 36 days cells were approaching the edge of the plasma clot. While there was a high cell density around the explant, it appeared that many of the more peripheral cells in the culture had floated loose from the central cell mass, and as so frequently occurs in old plasma cultures, had reanchored themselves on the surface of the peripheral clot in the flask. There they continued to proliferate and had established a sheet of cells covering nearly the whole of the plasma clot.

An attempt was made to fix and stain these plasma preparations but even using the highly selective staining procedure previously described (*6*) the plasma absorbed so much hematoxylin that the preparations were not satisfactory (fig. 1).

The set B cultures showed practically no migration or growth, even at 39 days of age (fig. 2).

Cultures of set C grew very poorly at first. At 16 days, however, in one culture the explant had succeeded in establishing a secure anchorage. By the thirty-third day there was a dense cell sheet adhering to the cellophane and glass around the explant. More peripherally loose cells liberated from this central cell mass had reanchored by attachment to cellophane and the glass of the flask and had then proliferated. When fixed and stained at 39 days there was a cell sheet over the whole 80 mm. diameter of the floor of the flask (fig. 3). Because of wrinkles in the cellophane, and because of the fact that in some areas the cells adhered to the glass floor of the flask and in other areas to the cellophane the design of the culture appeared irregular. Actually, however, growth, optical quality, and behavior on staining were far better in the cellophane cultures than in the plasma controls of set A.

In set-D cultures, each planted with 5 explants, some of the explants floated loose in the large volume of culture fluid used, were unable to

reanchor themselves and were sooner or later lost in changing the culture fluid. In each of the two cultures in this set, however, 2 or 3 explants did anchor (fig. 4). From these clumps cells were liberated into the culture fluid. Many of these cells in turn reanchored on the cellophane or the glass, so that cell growth in each of these cultures was rapid (figs. 4 and 5). When the cultures were fixed and stained at 39 days, growth was far more uniform and more luxuriant than in any of the preceding cultures.

In each of the two set-E cultures, many small fragments of the cell suspensions rapidly anchored in the culture flask. While it appeared that a great deal of the cell suspension was lost at the first fluid changes, the remainder proliferated with extreme rapidity. Many of the cells adhered to the cellophane, although in some areas adhesion was to the glass of the culture flask. At ten days, growth had been so rapid that it was feared continuance of the cultures would result in overloading the culture medium present. The cultures were therefore fixed and stained for study (fig. 6).

Experiment 2: The culture of set A, seeded from one Carrel flask appeared to grow very little if at all. By the fiftieth day it appeared that the amount of living cells in the culture had actually decreased. Many of the cells were free-floating and appeared to be disintegrating. The cultures from set B (fig. 7) grew well but not so luxuriantly as those of set C. Cultures of set C each seeded from 4 cultures grew rapidly and formed dense sheets of cell adherent to the cellophane. These even extended off the cellophane and spread onto the peripheral surface of the glass bottle. The growth of cultures of this set appeared to be far superior to that in culture of sets A and B.

While the growth of these cultures appeared extremely luxuriant it was still not uniform in certain areas which had apparently received a very light seeding owing to irregularities in the thickness of the floor of the flask. It was observed, furthermore, that following each change of fluid culture medium, the addition of chilled nutrient had knocked loose many cells from their substrate. These floated free in the fluid but disappeared from the fluid after a few hours. It appeared that they had again settled on the cellophane substrate and reattached themselves.

To confirm this observation and to determine if the reattached cells were growing satisfactorily, one culture in this set was shaken vigorously by hand for several minutes. As a result the culture fluid became cloudy with cells and small cell clumps shaken loose from the substrate. This culture was then returned to the incubator. When examined next day the supernatant fluid was practically clear, while the cellophane was covered with a dense cell sheet. The cells in this sheet were far more evenly distributed over the surface of the cellophane than formerly. There were even large and dense areas of cells on the glass of the flask. The appearance of one culture of this set fixed and stained at 79 days is shown in figure 8.

Culture of set D grew luxuriantly in the areas where the original cell suspension had settled most heavily, due to depressed areas in the floor of

the culture flask. These cultures were fixed and stained at the age of 39 days, when still apparently rapidly growing. One of these stained cultures is shown in figure 9.

Examination under the dissecting microscope identified some extracellular hematoxylin-staining material deposited on the surface of the cellophane. No detailed study was made of this material.

DISCUSSION

From data obtained it appears that at least 2 types of cells, one a freshly explanted chicken fibroblast, the other a mouse fibrosarcoma cell, can be easily grown on sheets of a cellophane substrate in Carrel flasks of 80 mm. diameter, and in one liter Blake bottles. The actual culture area obtained in these two types of container was approximately 45 square centimeters and 160 square centimeters respectively. The time required to obtain such cell sheets was 39 and 79 days respectively. From the behavior of these cultures there was no evidence whatsoever that the limit of practicable area for the plane cellophane substrate sheet culture had been reached. To the contrary the ease with which cell sheets of large size and satisfactory density were obtained indicates that the plane cellophane substrate culture can be carried to a far larger surface area of satisfactory cell density. Further, the information and experience gained in the handling of these large cultures studied have given an assurance that elimination of certain technical difficulties should allow the handling of such large cultures with far greater rapidity, ease and accuracy than has ever been possible with the more conventional types of culture used. The results obtained indicate that the methods of large culture growth should be fully explored in further experiments.

In the planting of these cultures two general methods were used. In the first, one or several discrete tissue explants were placed under the cellophane sheet. In the second a suspension of isolated cells and small cell clumps was pipetted into the culture flask. It appears that the former is the only method practicable with tissues freshly removed from the host animal and still in massive fragments. The use of a suspension of cells and small cell clumps has given such impressive preliminary results, however, that it is believed that the potentialities of this method for routine culture implantation and explantation should be exhaustively investigated.

In studies with the single cell, the data suggests that there is some growth relationship between the amount of living cells in the culture and the volume of medium to which these cells have free access. The same impression has been obtained in numerous studies on more usual size cultures, (*7*, *8*) in hanging-drop slides and in Carrel flasks. In the very large culture reported in this paper there was a continued suggestion of some necessary relationship between the amount of living cells used and the volume of culture medium to which they have free access. This is indicated in a number of instances in which a culture which had a relatively small inoculum of living cells showed unsatisfactory growth.

Until this factor can be more accurately defined and controlled, further exploration of the potentialities of massive tissue cultures should be made with care so that this factor does not unduly complicate data obtained.

There is no question, however, that the use of cellophane substrates poses many new problems in the handling of the cultures. For instance, in all these cultures, at each fluid change, there was some loss of cells, often severe, which had been shaken off the substrate and were free in the culture fluid. While this loss of cells can be reduced by reasonable precautions, it is still a very serious difficulty. Culture vessels so far tried have not adapted themselves well to overcoming this difficulty. Nor have these culture vessels allowed very satisfactory optical examination of the culture. It appears that for routine, facile use of the plane cellophane substrate culture, a new type of culture vessel is probably desirable.

SUMMARY AND CONCLUSION

Using fluid culture media of 40 percent horse serum, 20 percent 1:1 chick embryo extract, and 40 percent balanced salt solution, and using a substrate of sheet perforated cellophane, it has been possible to obtain cultures of 45 to 160 square centimeters in from 39 to 79 days.

It appears that the amount of tissue which may be grown in individual plane perforated cellophane substrate culture may be even further increased over that in any culture procedure yet tried.

The general ease of procedure developed makes the growth of such large cultures with these cell types already a practicable procedure, while the information obtained suggests even further simplification of method.

When the cells are freely accessible to the culture media, regardless of whether they are in small or large cultures, data obtained suggest that there is an apparently critical relationship in the amount of living cells to the volume of culture media.

The necessity of alterations in conventional culture methods and culture vessels to make more effective use of cellophane substrate methods is suggested.

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PLATE 139

FIGURE 1.—Thirty-nine-day-old plasma cultures of strain L mouse cells in a D–80 flask. The preparation has been fixed and stained in iron hematoxylin. The plasma has taken up so much stain that the preparations were not satisfactory. This culture belonged to set A, experiment 1.

40
30
20
10
0

PLATE 140

FIGURE 2.—Thirty-nine-day-old cellophane substrate culture of strain L cells in a D–80 flask, fixed and stained in iron hematoxylin. This culture belonged to set B of experiment 1 and received only 1 ml. of culture fluid. There is no evidence of either migration or proliferation.

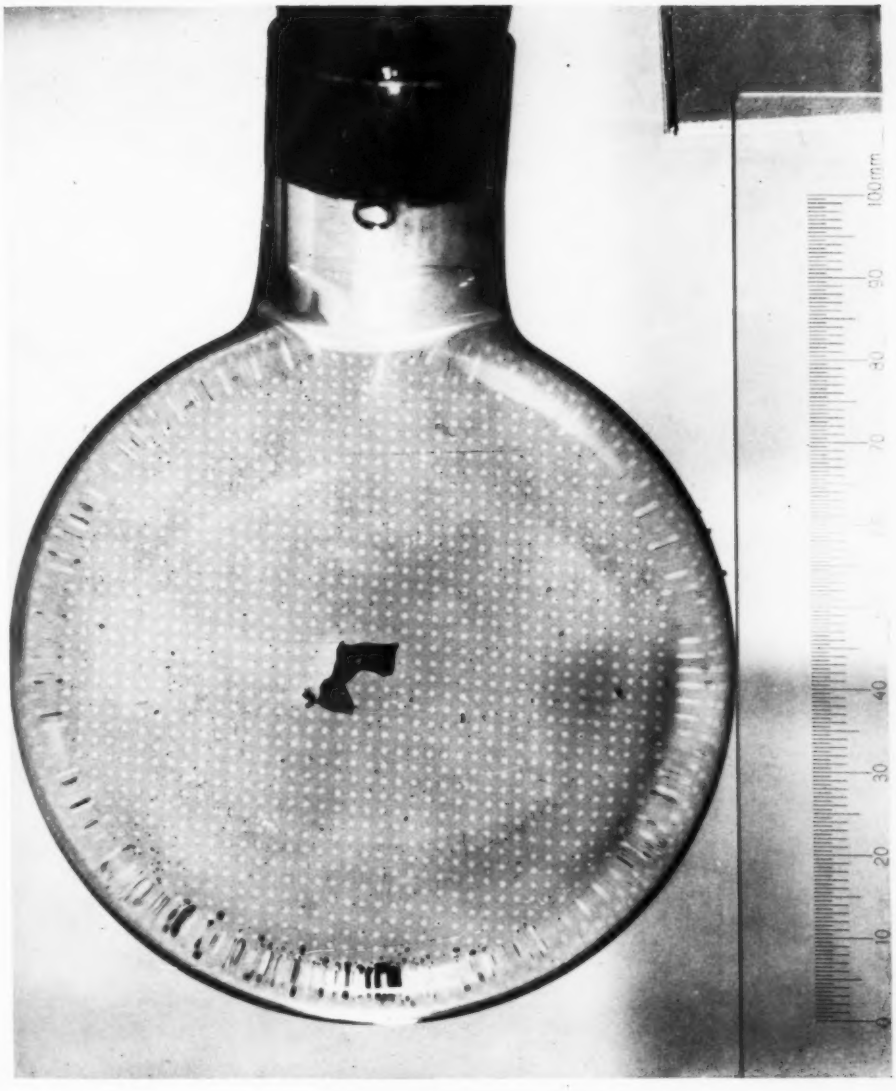
100 mm
90
80
70
40
30
20
10
0

PLATE 141

FIGURE 3.—Thirty-nine-day-old cellophane substrate culture of strain L cells in 5.5 ml. of culture fluid in a D–80 flask. The culture has been fixed and stained in iron hematoxylin. Grey areas with a clearly defined perforation design in white indicates stained cells adherent to the cellophane. Grey areas with the perforation design grey, too, indicate culture areas where the cells are also growing on the glass surface of the culture flask. This culture belonged to set C, experiment 1.

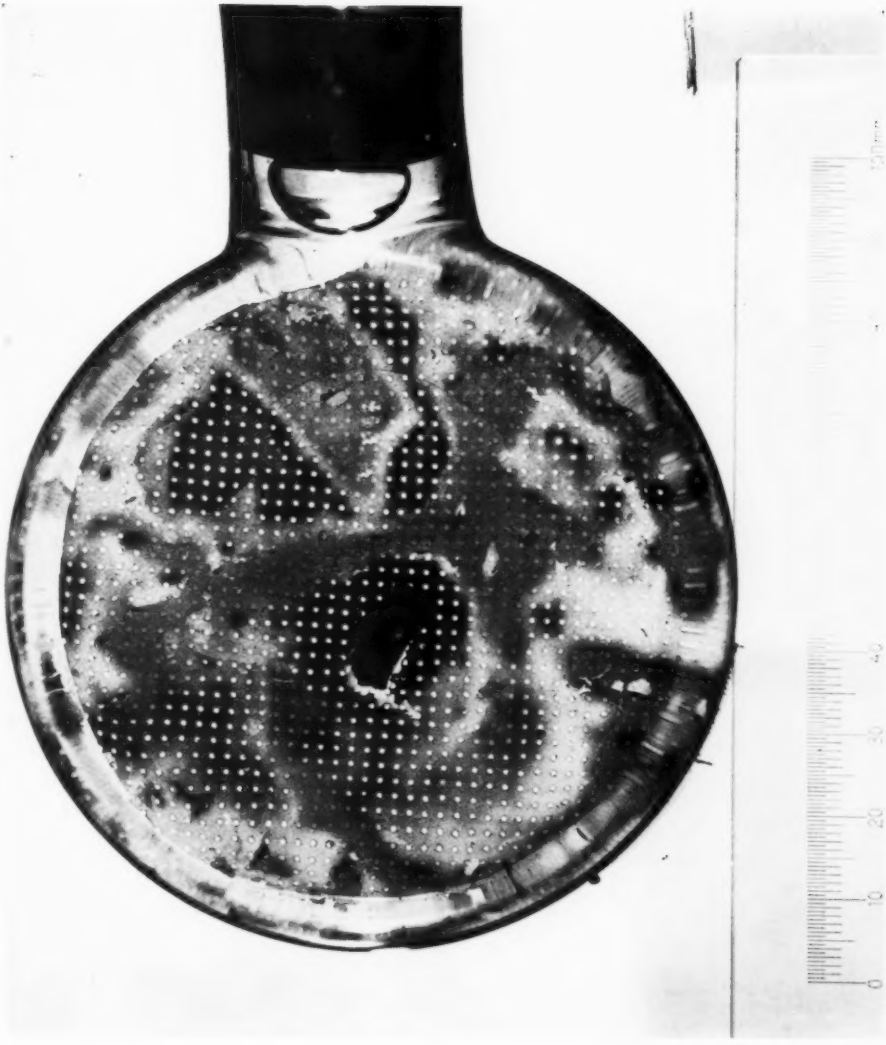

PLATE 142

FIGURE 4.—Thirty-nine-day-old cellophane culture of strain L cells. Originally 5 explants were used in the D–80 flask. 5.5 ml. of culture fluid was used. Three explants successfully anchored while the remaining two were lost in fluid changes. The ragged light area near the center resulted from tearing off an area of the cell sheet during staining. Note the excellent sheet of cells. The culture has been fixed and stained in iron hematoxylin. This culture belonged to set D, experiment 1.

0
10
20
30
40
50
60
70
80
90
100 mm

PLATE 143

FIGURE 5.—Thirty-nine-day-old cellophane culture of strain L cells. This culture was originally planted with 5 explants in a D–80 flask, of which only two have remained. The culture fluid volume was 5.5 cc. Note the uniformity and luxuriance of the cell sheet. The culture was fixed and stained in iron hematoxylin. The culture belonged to set D, experiment 1.

PLATE 144

FIGURE 6.—Ten-day-old cellophane cultures of strain L cells, D–80 flask. This culture was planted from cell suspension and received 5.5 ml. of culture fluid. The dark grey cell areas where the perforation design of the cellophane is obscured are usually areas of cells adhering to the glass of the culture flask and underlying the perforated cellophane sheet. The culture has been fixed and stained in iron hematoxylin. The culture belonged to set E, experiment 1.

100mm
90
80
70
60
50

PLATE 145

FIGURE 7.—Seventy-nine-day-old cellophane culture B, seeded with cell suspension from 2 Carrel D–3.5 cultures of chick heart fibroblasts. Note numerous cell colonies. The culture was fixed and stained in iron hematoxylin. The culture belonged to set B, experiment 2.

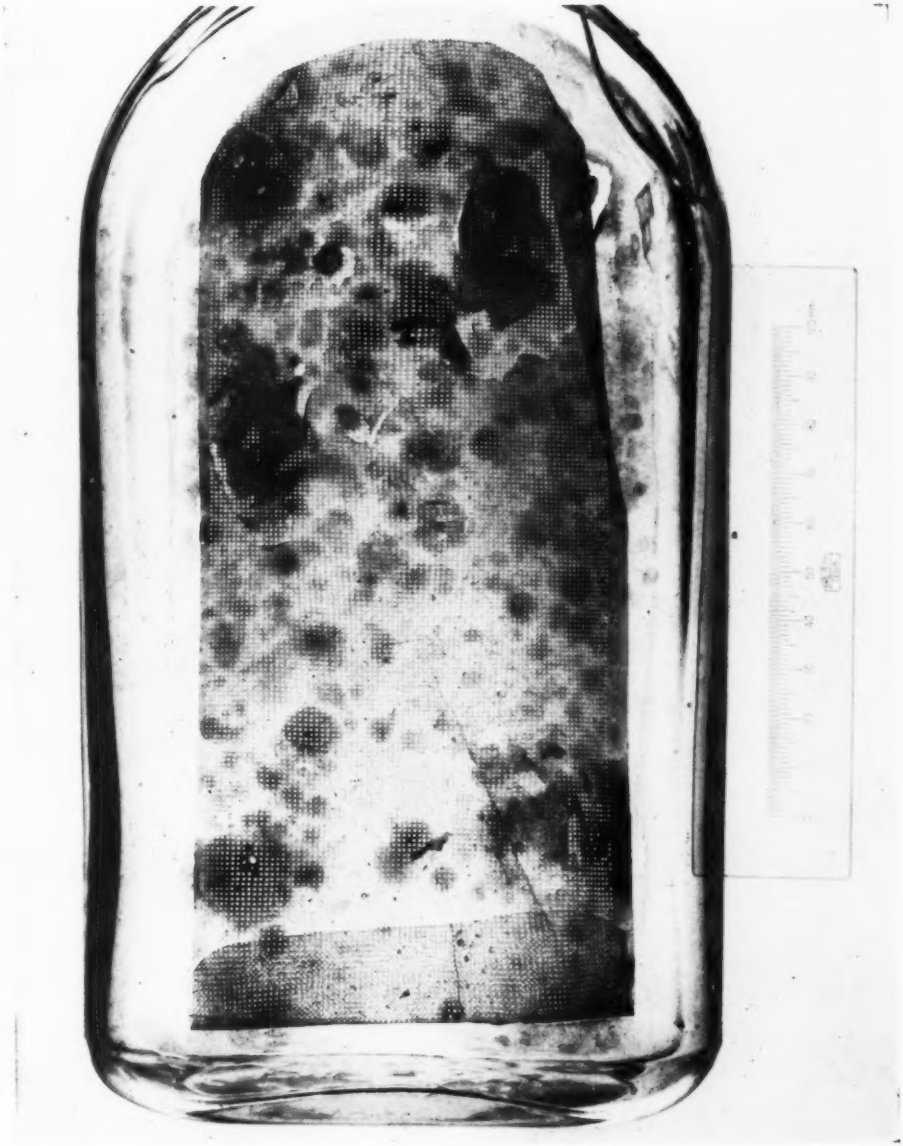

PLATE 146

FIGURE 8.—Seventy-nine-day-old cellophane culture, seeded with cell suspension from 4 D–3.5 cultures of chick heart fibroblasts. This culture was shaken several times during its history in order to get a more even distribution of cells. Note how the cells have grown out on the glass. The diffuse lighter area a little below the center of the cellophane sheet was apparently due to a raised area in the glass of the bottle floor. Many of the ragged fragments of cell sheet were pulled loose due to handling in staining and photographing. Fixed and stained in iron hematoxylin. The culture belonged to set C, experiment 2.

PLATE 147

FIGURE 9.—Thirty-nine-day-old cellophane culture prepared from a suspension of cells made up from 5 D–3.5 cultures of strain L cells. Note good cell density for this age culture. Note also lack of large cell clumps. Fixed and stained in iron hematoxylin. The culture belonged to set D, experiment 2.

INTRACELLULAR DISTRIBUTION OF ENZYMES. VI. THE DISTRIBUTION OF SUCCINOXIDASE AND CYTOCHROME OXIDASE ACTIVITIES IN NORMAL MOUSE LIVER AND IN MOUSE HEPATOMA [1]

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In previous reports (*4–9*) fractions were isolated by means of differential centrifugation from homogenates of rat liver and primary rat-liver tumors, and the distribution of enzymes and other biologically important compounds in these fractions was determined. These studies have now been extended to normal C3H mouse liver and a transplantable hepatoma (hepatoma 98/15) that arose spontaneously in a C3H mouse. This tumor appears to be an ideal tissue for comparative studies of this type because of its high degree of differentiation and its histologic resemblance to normal liver (*1*).

The present paper reports the distribution of succinoxidase and cytochrome oxidase activities in fractions isolated from normal C3H mouse liver and from hepatoma 98/15. Subsequent papers will describe the intracellular distribution of adenosinetriphosphatase, diphosphopyridine-nucleotide-cytochrome c reductase, isocitric dehydrogenase, and nucleic acids in these tissues.

MATERIALS AND METHODS

PREPARATION OF HOMOGENATES

Normal mouse liver was obtained from young adult mice (male and female) of the C3H strain. Hepatoma 98/15 was maintained by subcutaneous transplantation in C3H mice and the tumors were harvested 4–6 weeks after implantation. The tumors so obtained were 1.0 cm. or less in diameter, firm, and nonnecrotic.

The animals were killed by decapitation, bled, and the tissues removed and cooled over cracked ice. The tissues were weighed and homogenized in the apparatus of Potter and Elvehjem (*3*) with 9 volumes of 0.25 M sucrose. This sucrose concentration was chosen in preference to the higher sucrose concentrations required to maintain mitochondrial mor-

[1] Received for publication December 19, 1949.

phology (*2*) because of the greater ease of fractionation in 0.25 M sucrose coupled with the fact that some biochemical reactions are inhibited at the higher sucrose concentrations.

Centrifugal Fractionation of Homogenates

The homogenate was fractionated by differential centrifugation exactly as described in a previous publication (*11*). Five fractions were obtained: a nuclear fraction, N_w; a mitochondria fraction, M_{w2}; and a supernatant fraction S_1, which was in most cases further fractionated into a submicroscopic particle fraction, P_w; and a final supernatant or soluble fraction, S_2.

A brief description of the cytologic composition of the fractions is indicated at this point. The nuclear fraction, N_w, contained all the intact nuclei of the homogenate as well as any unbroken tissue cells and erythrocytes and some mitochondria that were not removed by the single washing to which this fraction was subjected. The mitochondrial fraction M_{w2} consisted almost entirely of mitochondria. In addition a small amount (less than 5 percent of the pellet volume) of the submicroscopic material was also detectable at the very top of the pellet when the mitochondria were sedimented. Little can be said with regard to the cytologic purity of the fractions P_w or S_2. Usually a small amount (less than 5 percent of the pellet volume) of mitochondrial material could be seen at the bottom of the submicroscopic particle pellet.

Enzymatic and Chemical Methods

Succinoxidase and cytochrome oxidase activities were determined as described by Schneider and Potter (*12*). In addition succinoxidase activity was also determined in the absence of added cytochrome c in the homogenates and mitochondrial fractions. The latter succinoxidase activity is apparently a measure of the availability to the succinoxidase system of cytochrome c *present* in the homogenate or mitochondria (*10, 11*).

Total nitrogen was determined by Nesslerization of micro-Kjeldahl digests (*13*).

RESULTS

The results of the centrifugal fractionation of normal C3H mouse liver and of hepatoma 98/15 are presented in tables 1, 2, and 3.

Distribution of Nitrogen

The nitrogen content of hepatoma 98/15 was about two-thirds that of normal mouse liver (table 1). This difference in the nitrogen content of the two tissues was accounted for almost entirely by great decreases in the amount of nitrogen present in the mitochondria and submicroscopic particles, M_{w2} and P_w, of the hepatoma. The amount of nitrogen present in the nuclear fractions of the two tissues was about the same while the nitrogen content of the supernatant, S_2, was somewhat lower in the hepatoma than in the normal mouse liver.

TABLE 1.—*The nitrogen content of mouse liver and hepatoma fractions*

[The total values reported are for 100 mg. of fresh tissue or an equivalent amount of each fraction and represent an average of 10 experiments with liver and 6 experiments with hepatoma]

Tissue fractions	C3H mouse liver		Hepatoma 98/15	
	Total nitrogen	Percent of total homogenate nitrogen	Total nitrogen	Percent of total homogenate nitrogen
	Mg.		*Mg.*	
Homogenate	3. 19 ±. 13	(100)	2. 30 ±. 075	(100)
N_w	. 570 ±. 071	17. 9	. 524 ±. 048	22. 8
M_{w2}	. 749 ±. 070	23. 5	. 300 ±. 015	13. 1
P_w	. 734 ±. 058	23. 1	. 450 ±. 036	19. 6
S_1	1. 20 ±. 070	37. 6	1. 06 ±. 039	46. 1

DISTRIBUTION OF SUCCINOXIDASE AND CYTOCHROME OXIDASE ACTIVITIES

The distribution of succinoxidase activity (table 2) and of cytochrome oxidase activity (table 3) was essentially the same in normal mouse liver and in hepatoma 98/15. The major portion of the activities of these enzyme systems present in the liver and hepatoma homogenates was recovered in the mitochondria fractions, M_{w2}, of these tissues. The enzymatic activities associated with the nuclear fractions, N_w, the submicroscopic particles, P_w, and the supernatant, S_1, can probably be explained by the presence of mitochondria in these fractions.

TABLE 2.—*Succinoxidase activities of mouse liver and hepatoma fractions*

[The total values reported are for 100 mg. of fresh tissue or an equivalent amount of each fraction and represents the average of 2 experiments with liver and 3 experiments with hepatoma]

Tissue fraction	C3H Mouse liver			Hepatoma 98/15		
	Succinoxidase activity			Succinoxidase activity		
	Total	Percent of total homogenate activity	Q_{O_2}	Total	Percent of total homogenate activity	Q_{O_2}
	$Mm.^3 O_2$ per hr.		$Mm.^3 O_2$ per hr. per mg. nitrogen	$Mm.^3 O_2$ per hr.		$Mm.^3 O_2$ per hr. per mg. nitrogen
Homogenate	4, 250	(100)	1, 340	755	(100)	325
N_w	842	19. 8	1, 650	128	17. 0	239
M_{w2}	2, 400	56. 5	3, 180	445	58. 9	1, 220
S_1	184	4. 3	91	58	7. 7	37
$N_w + M_{w2}$	3, 400	80. 0	----	640	84. 7	----
$N_w + S_1$	924	21. 7	----	151	20. 0	----
$M_{w2} + S_1$	3, 150	74. 1	----	611	80. 9	----
$N_w + M_{w2} + S_1$	4, 180	98. 4	----	703	93. 1	----
Homogenate minus cyt. c	2, 300	54. 1	----	693	91. 7	----
M_{w2} minus cytochrome c	1, 450	34. 1	----	308	40. 8	----

TABLE 3.—*Cytochrome oxidase activities of mouse liver and hepatoma fractions*

[The total values reported are for 100 mg. of fresh tissue or an equivalent amount of each fraction and each figure represents the average of 3 experiments]

Tissue fraction	C3H Mouse liver			Hepatoma 98/15		
	Cytochrome oxidase activity			Cytochrome oxidase activity		
	Total	Percent of total homogenate activity	Q_{o2}	Total	Percent of total homogenate activity	Q_{o2}
	Mm.^{3} O_2 per hr.		Mm.^{3} O_2 per hr. per mg. nitrogen	Mm.^{3} O_2 per hr.		Mm.^{3} O_2 per hr. per mg. nitrogen
Homogenate	6, 860	(100)	2, 060	1, 520	(100)	633
N_w	1, 360	19. 8	2, 440	195	12. 8	379
M_{w2}	5, 390	78. 6	6, 460	964	63. 4	3, 300
P_w	292	4. 1	351	247	16. 3	624
S_3	0	0	0	0	0	0

Although the distribution of succinoxidase and cytochrome oxidase was similar in the mouse liver and hepatoma, the *total* activity of these enzymes in the hepatoma and hepatoma fractions was much lower than in the liver or liver fractions. The *specific* activity (Q_{o2}) of the hepatoma homogenate and fractions was also lower than that of the liver homogenate and liver fractions with one exception. The cytochrome oxidase Q_{o2} of the hepatoma submicroscopic particles was considerably higher than that of the corresponding liver fractions.

It is of especial interest to compare the enzymatic activities of the liver and hepatoma mitochondria because this fraction is essentially cytologically homogeneous and also because the major portion of the succinoxidase and cytochrome oxidase activities of both tissues was recovered in this fraction. When such a comparison is made, it is noted that the *total* succinoxidase and cytochrome oxidase activity of the liver mitochondria was about 5.5 times that of the hepatoma mitochondria while the *specific* activity of the liver mitochondria was only 2 to 2.5 times that of the hepatoma mitochondria. Thus it would appear that the decreased succinoxidase and cytochrome oxidase activity of hepatoma as compared to normal liver could be attributed *in part but not entirely* to the decreased amount of mitochondrial material in the hepatoma.

The succinoxidase activities of the homogenates and mitochondria in the absence of added cytochrome c are also given in table 2. The omission of added cytochrome c resulted in a considerable decrease in the succinoxidase activities of the liver homogenate and of the liver and hepatoma mitochondria but not of the hepatoma homogenate. Since previous studies have demonstrated that the succinoxidase activity in the absence of added cytochrome c is a measure of the availability of cytochrome c present in a tissue suspension (*10, 11*), it would appear that the isolation of the hepatoma mitochondria resulted in a decreased availability of the cytochrome c present in the hepatoma mitochondria.

It will be noted that the sum of the succinoxidase activities of the frac-

tions N_w, M_{w2}, and S_1 was only about 80 percent of the activity of the original homogenate (table 2). A similar situation occurred in other studies in this laboratory with rat liver (*11*). Since this recovery of succincxidase activity was much lower than noted previously with fractions obtained from rat liver, rat liver tumors, and rat kidney (*7–9*), studies in which the three fractions were recombined in various permutations and combinations were made. When this was done (table 2) it was noted that the supernatant fraction, S_1, augmented the succinoxidase activity of the mitochondria considerably but that it was necessary to recombine all three fractions in order to recover the full activity of the homogenates. The recovery of cytochrome oxidase activity in the fractions N_w, M_{w2}, and P_w was greater than 90 percent of the homogenate activity and no attempt was made to determine the effect on cytochrome oxidase activity of the recombination of fractions.

DISCUSSION

The distribution of nitrogen in the normal C3H mouse liver and in hepatoma 98/15 was somewhat different than that of the rat liver and rat-liver tumors. The amount of nitrogen recovered in the nuclear fraction of hepatoma 98/15 was about the same as that in the nuclear fraction of mouse liver (table 1). In the case of the rat-liver tumor however, the dry weight and protein content of the nuclear fraction were much greater than that of the nuclear fraction of rat liver (*4–9*).

The nitrogen content of the submicroscopic particles isolated from hepatoma 98/15 was much lower than that of the submicroscopic particles obtained from normal mouse liver (table 1). This finding was similar to that of Price *et al.* (*4–6*). These authors found that the *protein* content of the submicroscopic particles obtained from rat-liver tumors was considerably lower than that of the submicroscopic particles isolated from normal rat liver.

In the case of the mitochondrial fraction, the present results (table 1) are in complete agreement with those obtained with rat liver and rat-liver tumors (*4–9*). The amount of mitochondrial material obtained from hepatoma 98/15 and from rat-liver tumors was much less than that obtained from C3H mouse liver and from rat liver, respectively.

The distribution of succinoxidase and cytochrome oxidase activity in C3H mouse liver and in hepatoma 98/15 (tables 2 and 3) was essentially the same as in rat liver and in rat-liver tumor (*7–9.*) The major portion of the succinoxidase and cytochrome oxidase activities of these tissues was recovered in the mitochondria isolated from these tissues.

SUMMARY

Homogenates of normal C3H mouse liver and of hepatoma 98/15 were fractionated by means of differential centrifugation into a nuclear, a mitochondrial, a submicroscopic particulate, and a supernatant or soluble fraction. The distribution in these fractions of total nitrogen and of succinoxidase and cytochrome oxidase activities was determined.

The total nitrogen content of the hepatoma homogenate was about two thirds that of normal liver. This difference in nitrogen content was accounted for in large part by the lower nitrogen contents of the hepatoma mitochondria and submicroscopic particles. The nitrogen content of the liver and hepatoma nuclear fractions was about the same, whereas the nitrogen content of the hepatoma supernatant was somewhat less than that of the liver supernatant.

The succinoxidase and cytochrome oxidase activities of the hepatoma homogenate were only about one fifth as great as the succinoxidase and cytochrome oxidase activities of the liver homogenate. The distribution of these enzymatic activities was, however, essentially the same in both tissues; the major portion of the succinoxidase and cytochrome oxidase activities of the homogenates was associated with the mitochondria fractions.

These results are compared with the results obtained in previous studies with rat liver and rat-liver tumors.

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INTRACELLULAR DISTRIBUTION OF ENZYMES. VII. THE DISTRIBUTION OF NUCLEIC ACIDS AND ADENOSINETRIPHOSPHATASE IN NORMAL MOUSE LIVER AND MOUSE HEPATOMA[1]

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The preceding paper of this series (*16*) reported the distribution of total nitrogen and of succinoxidase and cytochrome oxidase activities in fractions obtained from normal C3H mouse liver and from a transplantable mouse hepatoma. The present report describes the distribution of desoxypentose nucleic acid (DNA), pentose nucleic acid (PNA), and adenosinetriphosphatase (ATP-ase) activity in similar fractions obtained from these tissues.

MATERIALS AND METHODS

Homogenates of normal C3H mouse liver and of hepatoma 98/15 in 0.25 M sucrose were made as was described in the preceding report (*16*). The centrifugal fractionation of the homogenates was carried out exactly as described previously (*15*) and resulted in the isolation of four fractions: a nuclear fraction, N_w, containing all the intact nuclei of the homogenate as well as a few unbroken cells and some mitochondria; a mitochondrial fraction, M_{w2}, consisting essentially of mitochondria; a submicroscopic particle fraction, P_w; and a supernatant or soluble fraction, S_2.

Nucleic acids were extracted from the tissue fractions by a modification of an earlier method (*10*). Perchloric acid was substituted for the trichloroacetic acid previously used. The tissue fractions were extracted once with cold 12 percent perchloric acid (made by adding 0.2 volume of 60 percent Merck reagent perchloric acid) and twice with cold 6 percent perchloric acid to remove acid soluble compounds (*e. g.*, nucleotides, sucrose). The tissue residues were extracted 3 times with commercial absolute ethanol to remove phospholipids and were then heated 15 minutes at 90° C. with 6 percent perchloric acid to extract the nucleic acids. The extracted DNA was estimated colorimetrically by means of the diphenylamine reaction and PNA by means of the orcinol reaction.

ATP-ase activity was measured in the presence of calcium ions as described by DuBois and Potter (*3*). The assays were made at two levels of tissue and the amounts of each fraction assayed were adjusted to yield an enzymatic activity approximating that of the homogenate.

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RESULTS

The DNA and PNA contents of normal C3H mouse liver fractions and of hepatoma 98/15 fractions are reported in table 1. The ATP-ase activities of these fractions are presented in table 2.

TABLE 1.—*Desoxypentose nucleic acid (DNA) and pentose nucleic acid (PNA) content of mouse liver and hepatoma fractions*

(The total values reported are for 100 mg. of fresh tissue or an equivalent amount of each fraction and represent the average of 3 experiments)

Tissue fraction	C3H Mouse liver						Hepatoma 98/15					
	DNA phosphorus			PNA phosphorus			DNA phosphorus			PNA phosphorus		
	Total	Percent of total homogenate DNA phosphorus	Per mg. N	Total	Percent of total homogenate PNA phosphorus	Per mg. N	Total	Percent of total homogenate DNA phosphorus	Per mg. N	Total	Percent of total homogenate PNA phosphorus	Per mg. N
	Micrograms		*Micrograms*	*Micrograms*		*Micrograms*	*Micrograms*		*Micrograms*	*Micrograms*		*Micrograms*
Homogenate	27. 9	(100)	8. 4	92. 9	(100)	28. 1	31. 6	(100)	13. 7	97. 5	(100)	42. 4
N_w	22. 4	80. 3	42. 3	10. 2	11. 0	19. 2	25. 3	80. 0	50. 8	16. 0	16. 4	32. 2
M_{w2}	1. 55	5. 6	1. 8	15. 6	16. 8	18. 4	1. 09	3. 5	3. 3	10. 3	10. 6	30. 9
P_w	3. 96	14. 2	5. 2	48. 7	52. 4	64. 2	4. 65	14. 7	10. 1	38. 8	39. 8	84. 0
S_2	-----	-----	----	15. 3	16. 5	12. 0	-----	-----	----	25. 7	26. 4	24. 5

TABLE 2.—*Adenosinetriphosphatase (ATP-ase) activities of mouse liver and hepatoma fractions*

(The total values reported are for 100 mg. of fresh tissue or an equivalent amount of each fraction and represent the average of 2 experiments)

Tissue Fraction	C3H Mouse liver			Hepatoma 98/15		
	ATP-ase activity			ATP-ase activity		
	Total [1]	Percent of total homogenate	Q [2]	Total [1]	Percent of total homogenate	Q [2]
Homogenate	1, 580	(100)	485	907	(100)	379
N_w	495	31. 3	822	342	37. 8	606
M_{w2}	790	50. 0	1, 050	117	12. 9	392
P_w	240	15. 2	316	318	35. 1	690
S_2	80	5. 1	63	125	13. 8	122
ΣN_w, M_{w2}, P_w, S_2	1, 605	--------	--------	902	--------	--------

[1] Micrograms inorganic phosphorus liberated from ATP in 15 minutes by 100 mg. fresh tissue or an equivalent amount of each fraction.
[2] Micrograms inorganic phosphorus liberated in 15 minutes per mg. nitrogen.

DISTRIBUTION OF DNA

The distribution of the nuclear nucleic acid, DNA, in normal mouse liver and mouse hepatoma fractions is given in table 1. The results show that the DNA content of the hepatoma (31.6 micrograms DNA phosphorus) was only slightly higher than that of the normal liver (27.9 micrograms). About 80 percent of the DNA of both tissues was recovered in the nuclear fraction of these tissues. In previous studies with rat liver,

rat kidney and rat-liver tumors, DNA was recovered almost quantitatively in the nuclear fractions of these tissues (*6*, *7*, *9*, *12*, *13*, *14*). Price *et al.* (*8*) however observed low recoveries of DNA in the nuclear fraction of rat-liver tumors and found that these tumors contained extracellular Feulgen positive particles of varying size. This explanation would not appear to account for the present results inasmuch as poor recoveries of DNA were obtained in the nuclear fractions of normal liver as well as hepatoma. Extracellular DNA containing (Feulgen positive) particles would not be expected to be present in *normal* mouse liver. Other possibilities that must be considered in seeking an explanation for the low recovery of DNA in the nuclear fraction are: *a*) the nuclei of mouse tissues may be more fragile than those of the rat tissues previously studied (*6–9*, *12–14*) and were partially fragmented during the homogenization process, or *b*) some of the nuclei were disrupted when the homogenates were forced through the hypodermic syringe needle. The latter step was not used in previous studies and would appear to be the more likely explanation for the low recoveries observed. It is of some interest to note that about 14 percent of the DNA of both normal mouse liver and mouse hepatoma was recovered in the submicroscopic particulate fraction of these tissues (table 1).

Distribution of PNA

The PNA content of normal C3H mouse liver (92.9 micrograms PNA phosphorus) was slightly lower than that of the hepatoma (97.5 micrograms). The distribution of PNA was essentially the same in both tissues (table 1). PNA was present in all fractions but only in one fraction was the concentration of PNA (micrograms PNA phosphorus per mg. nitrogen) greater than in the original homogenate. This fraction was the submicroscopic particulate fraction, P_w, and in this fraction 52.4 percent of the normal liver PNA and 39.8 percent of the hepatoma PNA was recovered. These data agree with previous data obtained with rat tissues (*6–9*). The supernatant or soluble fraction (S_2) of the hepatoma contained considerably larger amounts of PNA than did the soluble fraction of normal liver. This agrees with the observation of Brachet and Jeener (*2*) that rapidly growing tissues contained larger amounts of nonsedimentable PNA than differentiated tissues.

Distribution of ATP-ase

The ATP-ase activities of mouse liver and hepatoma fractions are reported in table 2. The ATP-ase activity of the hepatoma homogenate (907 micrograms phosphorus) was about 60 percent as great as that of the liver homogenate (1,580 micrograms). This difference was accounted for by the greatly decreased activity of the hepatoma mitochondria. The total activity of the latter was only 117 as compared to 790 for the liver mitochondria. The total ATP-ase activity of the other hepatoma fractions was not strikingly different from that of the corresponding liver fractions.

The distribution of ATP-ase activity was much different in the hepatoma than in normal mouse liver. In the latter tissue, the largest percentages of the total homogenate ATP-ase activity were recovered in the mitochondrial and nuclear fractions, while in the case of the hepatoma the largest percentages were recovered in the nuclear and submicroscopic particulate fractions.

The distribution of ATP-ase activity in normal mouse liver and mouse hepatoma fractions (table 2) was similar to that observed previously with normal rat liver and rat-liver tumors (*12*, *13*). The present results differ from the results obtained with the rat tissues in one respect. In the case of the rat, the total ATP-ase activity of the liver and liver tumor were about the same, whereas the present results show that the total ATP-ase activity of mouse liver was much higher than that of mouse hepatoma. The total activity of the latter was about the same as that of rat liver and rat-liver tumors (*12*, *13*).

DISCUSSION

The DNA content of hepatoma 98/15 was much lower than that of rat-liver tumors as well as of a variety of other rat and mouse tumors studied previously (*11*, *13*, *17*). In the case of the rat-liver tumor, several lines of evidence suggested that the higher DNA content of rat-liver tumors as compared to normal rat liver was due to a relative decrease in the amount of cytoplasm in the tumor as shown by the following facts: (a) normal rat liver and rat-liver tumors had the same size distribution of nuclear volumes (*1*), (b) the ratio of cytoplasmic volume to nuclear volume was much lower for the rat-liver tumor than for rat liver (*11*), and (c) the dry weight and protein content of the nuclear fraction of rat-liver tumors was much greater than that of the nuclear fraction of rat liver (*8*, *13*). In contrast to the rat liver—rat-liver tumor studies—the present results show that the DNA contents of C3H mouse liver and of hepatoma 98/15 were about the same (table 1). This fact coupled with the finding that the nuclear fractions of both C3H mouse liver and of hepatoma 98/15 contained about the same amounts of total nitrogen (*16*) would appear to indicate that the relative amounts of cytoplasm in these two tissues were about the same.

Price *et al.* (*7–9*) and Griffin *et al.* (*4*) showed that the increased DNA content of rat-liver tumors was intimately related to the carcinogenic process. They demonstrated that the DNA content of rat liver increased when the rats were fed carcinogenic azo dyes for short periods of time. No increased DNA content was observed when weak or mildly carcinogenic azo dyes were fed for similar periods of time. This increase in the DNA content of rat liver would appear to be a special manifestation of this type of carcinogen, however, rather than of general significance to the carcinogenic process in view of the present results (table 1) as well as those of Griffin *et al.* (*5*). The latter authors found that the DNA content of rat-liver tumors induced with acetoaminofluorene was about the same as that of control rat livers.

The distribution of PNA in the mouse liver and mouse hepatoma fractions (table 1) was somewhat different from that obtained with rat liver and rat-liver tumors (*7–9*). Whereas the ratios of the PNA contents of the fractions M_{w2}, P_w and S_2 were about 1:3:1 for mouse liver and 1:4:2.6 for hepatoma 98/15 (table 1) the corresponding ratios for rat liver and rat-liver tumors were 1:1:1 and 1:2:4.5, respectively (*7–9*). The reason for these discrepancies is not known. However, the results with all tissues show that PNA is concentrated in only 1 fraction, the submicroscopic particles, P_w. The results with both the rat and the mouse also show that the fractions M_{w2} and P_w of the tumor contain less PNA than the corresponding liver fractions and that the PNA content of the tumor supernatant, S_2, is greater than that of the liver supernatant.

Although the distribution of ATP-ase activity was essentially the same in mouse liver as in rat liver and in mouse hepatoma as in rat-liver tumors, the present results with mouse tissues do show differences from those obtained with the rat tissues. In the first place, the ATP-ase activity of mouse liver was considerably greater than that of hepatoma 98/15 (table 2), whereas the activities of rat liver and rat-liver tumor were about the same (*13*). The transformation from normal liver to liver tumor in both mouse and rat resulted in great decreases in the ATP-ase activity of the isolated mitochondrial fractions. In the rat, however, the decreased activity of the liver tumor mitochondria was accompanied by a corresponding increase in the activity of the liver tumor fraction containing the submicroscopic particles, while in the case of the mouse the decreased activity of the hepatoma mitochondria was not accompanied by increased activity of the hepatoma submicroscopic particles.

SUMMARY

C3H mouse liver and hepatoma 98/15 were fractionated by means of differential centrifugation into nuclear, mitochondrial, submicroscopic particulate and supernatant or soluble fractions. The distribution in these fractions of desoxypentose nucleic acid (DNA), pentose nucleic acid (PNA), and adenosinetriphosphatase (ATP-ase) activity was studied.

The DNA content of mouse liver was about the same as that of the hepatoma. Approximately 80 percent of the DNA of both tissues was recovered in the nuclear fractions of these tissues.

The PNA content of mouse liver was also about the same as that of the hepatoma. Approximately 52 percent of the PNA of mouse liver and 40 percent of the PNA of the hepatoma was recovered in the submicroscopic particulate fraction.

The ATP-ase activity of the hepatoma was about 60 percent as great as that of mouse liver. This difference between the activities of mouse liver and hepatoma was largely accounted for by the low activity of the hepatoma mitochondria. The major portions of the ATP-ase activity of mouse liver were associated with the mitochondrial and nuclear fractions while in the hepatoma the nuclear and submicroscopic particulate fractions contained the largest portion of the total ATP-ase activity.

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INTRACELLULAR DISTRIBUTION OF ENZYMES. VIII. THE DISTRIBUTION OF DIPHOSPHOPYRIDINE NUCLEOTIDE—CYTOCHROME C REDUCTASE IN NORMAL MOUSE LIVER AND MOUSE HEPATOMA[1]

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A preceding paper of this series (*1*) reported the distribution of two cytochrome-linked respiratory enzyme systems, succinoxidase and cytochrome oxidase, in cell fractions obtained from normal C3H mouse liver and from a transplantable mouse hepatoma. The present paper reports the distribution in similar fractions derived from the two tissues of DPN (diphosphopyridine nucleotide)—cytochrome c reductase, a third cytochrome-linked system that is extremely active in the enzymatic reduction of cytochrome c.

METHODS

Preparation of the homogenates of normal C3H mouse liver and hepatomas 98/15 in 0.25 M sucrose and fractionation of the homogenates by means of differential centrifugation were carried out exactly as described in previous papers of this series (*1*, *2*). Estimations of DPN-cytochrome c reductase activity and of total nitrogen were made on the original homogenates and on the following four fractions: nuclei, (N_w); mitochondria (M_{w2}); submicroscopic particles, (P_w); and the final supernatant, (S_2).

Determination of the DPN-cytochrome c reductase activity of the preparations was carried out at 24° C. according to an assay method similar in most respects to that described in an earlier publication (*3*). The reaction mixtures were made up by combining the following reagents, in order: 0.30 ml. of 0.5 M potassium phosphate buffer (pH 7.4), 0.10 ml. of 0.82 M nicotinamide, water to give a final volume of 3.00 ml., 0.30 ml. of 2×10^{-3} M KCN, 0.050 to 0.200 ml. of a suitable dilution of each enzyme preparation, 0.40 ml. of 2.5×10^{-4} M oxidized cytochrome c, and 0.30 ml. of 1.4×10^{-3} M dihydrodiphosphopyridine nucleotide ($DPNH_2$).

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On addition of $DPNH_2$, the reaction mixtures were quickly transferred to cuvettes and the increase in optical density at 550 mμ followed at 1 minute intervals in the Beckman quartz spectrophotometer. The total activity of the preparations was expressed as micromoles cytochrome c reduced per minute by 100 mg. of whole tissue or an equivalent amount of each fraction, and the specific activity (Q) as micromoles cytochrome c reduced per minute per mg. of total nitrogen of each preparation.

Cytochrome c was obtained from the Treemond Co. In order to maintain the substance in the oxidized state, the aqueous solution of cytochrome c was made 0.005 N with respect to HCl. On reduction with sodium hydrosulfite, the increase in optical density at 550 mμ of the solution corresponded to molecular extinction coefficients of 0.90×10^7 sq. cm. per mole for oxidized cytochrome c and 2.87×10^7 sq. cm. per mole for reduced cytochrome c.

$DPNH_2$ was prepared from samples of DPN purified by countercurrent distribution (*4*) and containing 90 to 98 percent DPN. Eight to twelve mg. of DPN were dissolved in 2.0 ml. of a freshly prepared solution of sodium hydrosulfite in 0.10 M K_2HPO_4 (2.0 mg. $Na_2S_2O_4$ per ml.); the mixture was heated for 1 minute at 100° C., quickly chilled in an ice bath, diluted with 0.1 M K_2HPO_4 to give a final $DPNH_2$ concentration of 1.5×10^{-3} M, and aerated for 10 minutes. When kept at 0° C. the $DPNH_2$ solution was stable for at least 6 hours; aliquots of the solution were neutralized to pH 7.4 with 0.5 M KH_2PO_4 immediately before use in the DPN-cytochrome c reductase determination. The concentration of $DPNH_2$ in the neutralized solution was 1.4×10^{-3} M on the basis of an extinction coefficient at 340 mμ of 6.27×10^6 sq. cm. per mole (*5*) for the pure compound.

Determination of total nitrogen was carried out colorimetrically after acid digestion of the samples (*6*).

RESULTS

The distribution of DPN-cytochrome c reductase among fractions obtained from homogenates of C3H mouse liver and hepatoma 98/15 is shown in table 1. The validity of the method of enzyme assay for both tissues is indicated by the fact that the total activity recovered in the four fractions closely approximated the amount originally present in the homogenates.

In the case of normal liver practically all the activity was present in the three sedimentable fractions, nuclei (N_w), mitochondria (M_{w2}), and submicroscopic particles (P_w), the last 2 fractions containing most of the activity. As shown by the specific activity (Q) values, the enzyme was concentrated in submicroscopic particles to a considerable degree and only slightly in mitochondria. Although these findings are in general similar to results previously obtained with normal rat liver (*3*), the specific activities of the mouse-liver homogenate, mitochondria, and submicroscopic particles are, however, lower than the activities reported earlier for corresponding rat-liver preparations. That this is probably a species

difference and is not due to present modifications in the method of fractionation or of enzyme determination is indicated by the fact that recent assays of rat-liver fractions in this laboratory have yielded specific activities in agreement with the earlier data.

TABLE 1.—*DPN (diphosphopyridine nucleotide)-cytochrome c reductase activities of mouse liver and hepatoma fractions. The total values reported are for 100 mg. of fresh tissue or an equivalent amount of each fraction and are the average of 2 experiments*

Tissue Fraction	C3H Mouse liver			Hepatoma 98/15		
	DPN-Cytochrome c reductase activity			DPN-Cytochrome c reductase activity		
	Total [1]	Percent of total homogenate	Q [2]	Total [1]	Percent of total homogenate	Q [2]
Homogenate	6. 95	(100)	2. 14	8. 80	(100)	3. 82
N_w	. 63	9. 1	1. 10	1. 30	14. 8	2. 54
M_{w2}	1. 97	28. 3	2. 56	2. 40	27. 3	7. 48
P_w	4. 12	59. 3	5. 49	4. 56	51. 8	9. 22
S_2	. 24	3. 5	. 19	. 69	7. 8	. 65
ΣN_w, M_{w2}, P_w, S_2	6. 96			8. 95		

[1] Micromoles cytochrome c reduced by 100 mg. fresh tissue or an equivalent amount of each fraction.
[2] Micromoles cytochrome c reduced per minute per mg. nitrogen.

As shown in table 1, the hepatoma homogenate and all fractions derived from it showed somewhat more total enzyme activity and considerably higher specific enzyme activity than the corresponding preparations obtained from normal liver. In a consideration of the activities of the individual fractions, it may be noted that the specific activity of hepatoma mitochondria was approximately twice that of the hepatoma homogenate, whereas the specific activity of liver mitochondria was greater than that of the liver homogenate by a factor of only 1.2. The extent of concentration of the enzyme system in submicroscopic particles, however, was nearly the same for both tissues.

The finding that the specific activity of the nuclear fractions of both tissues was considerably lower than that of the homogenates suggests that nuclei themselves contain little if any DPN-cytochrome c reductase. This interpretation is based on the fact that the nuclear fraction is not homogeneous but contains intact cells and free mitochondria in amounts probably sufficient to account for the activity observed. It may also be mentioned that resedimentation of the submicroscopic particles from both tissues did not yield a firmly packed pellet; it therefore seems likely that the relatively low but appreciable activity of the supernatants (S_2) was due to a small amount of submicroscopic particulate material still remaining in suspension after the final centrifugation.

DISCUSSION

In a previous paper of this series (*1*), it was shown that hepatoma 98/15 is deficient to a considerable extent when compared with normal liver in two important respiratory enzyme systems, succinoxidase and cytochrome oxidase. The specific activity of hepatoma homogenates was one-third

to one-fourth that of liver homogenates and the activity of hepatoma mitochondria approximately one-half that of liver mitochondria. In the case of both hepatoma and normal liver, by far the greatest proportion of the respiratory enzyme systems was concentrated in mitochondria. In other investigations (*2*) it was found that adenosinetriphosphatase (ATP-ase), an enzyme also concentrated in normal liver mitochondria, was present in hepatoma mitochondria in a much lower concentration.

It is of interest that DPN-cytochrome c reductase, in contrast to the respiratory enzymes and ATP-ase, is present in hepatoma 98/15 in *greater* amount than in normal liver and was thus apparently retained by the liver cell in the transition from the normal to the neoplastic state. Furthermore, the hepatoma fraction showing a large relative increase in DPN-cytochrome c reductase activity, namely the mitochondria, was the site of decreased respiratory enzyme and ATP-ase activity.

In previous experiments with rat liver (*3*), it was found that the distribution of DPN-cytochrome c reductase among cell fractions was somewhat similar to the distribution of pentose nucleic acid (PNA), both the enzyme and the nucleic acid being largely sedimentable and concentrated in submicroscopic particles. The present data (table 1), together with the results of a recent study of the distribution of PNA in homogenates of mouse liver and hepatoma 98/15 (*2*), show that a similar situation also holds for the latter 2 tissues. Furthermore, in a comparison of normal liver and hepatoma, it may be noted that the increased DPN-cytochrome c reductase concentration in the hepatoma and the fractions derived from it (table 1) is accompanied by an increased PNA concentration (*2*). Although the extent of increase of the 2 properties in the hepatoma is not identical, these findings again bring up the interesting question of a possible relationship between PNA and DPN-cytochrome c reductase.

SUMMARY

A study is reported of the distribution of DPN (diphosphopyridine nucleotide)-cytochrome c reductase in cell fractions obtained by differential centrifugation of homogenates of C3H mouse liver and hepatoma 98/15.

In both tissues most of the enzyme activity was recovered in 2 sedimentable fractions, mitochondria and submicroscopic particles. In the case of normal liver, the enzyme was concentrated, in terms of total nitrogen, to a considerable degree in submicroscopic particles and slightly in mitochondria. The specific enzyme activities of the hepatoma homogenate and all fractions derived from it were higher than the activities of the corresponding preparations obtained from liver. In the hepatoma the enzyme system was also concentrated in both mitochondria and submicroscopic particles, but the extent of concentration in mitochondria was much greater than in the mitochondria of normal liver.

The findings are discussed in relation to previous similar studies of the intracellular distribution of succinoxidase, cytochrome oxidase, adenosinetriphosphatase, and pentose nucleic acid.

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OBSERVATIONS ON INBRED MICE EXPOSED TO DDT [1]

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The use of DDT (1,1,1-trichloro-2,2-bis (p-chlorophenyl)-ethane) as an insecticide began in 1939 (*1*), and by 1947 annual production in the U. S. had reached 49.6 million pounds (*2*). Pharmacologic studies have shown that the chief systemic actions of DDT in animals are (a) central nervous system effects characterized by hyperexcitability, generalized tremors, spastic or flaccid paralysis and convulsions, and (b) hyaline degeneration and focal necrosis of the liver (*3*, *4*). The literature to 1947 has been reviewed by Stammers and Whitfield (*5*).

Andervont (*6*) administered technical (95 percent pure) DDT, dissolved in olive oil, orally and subcutaneously to 85 male and female mice of strains A, C, and C_{57} black. Single and multiple doses were given, and the mice were observed 6–12 months following treatment. No animal developed a tumor that could be considered to be induced by the DDT.

Fitzhugh and Nelson (*7*) reported that the liver damage produced in rats by feeding DDT for 18 months was followed by the development of "hepatic cell tumors" in a small percentage of the animals. No other reports have been found on the carcinogenicity of this widely used compound (*8*). Data are presented below from an experiment designed to demonstrate the possible carcinogenicity of DDT in inbred mice.

EXPERIMENTAL

A 5 percent solution of DDT in kerosene (issued by the Office of the Quartermaster General, U. S. Army, and described as "Insecticide, spray, DDT, residual effect; ingredients 5 percent DDT, 95 percent kerosene") was used to paint strain C [3] mice of both sexes at weekly intervals for 52 weeks. The mice were painted over the interscapular area with a #6 camel-hair brush starting at 2 months of age. Control mice were not painted.

During the exposure period many of the painted animals showed tremors

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[3] Designated "B alb C" in (*9*).

and hyperexcitability. Patchy epilation and roughening of the skin at the site of painting were also noted.

Two to three weeks after the 52nd painting the 14 experimental and 16 control animals were killed by ether inhalation. Sections from all organs and the painted area were fixed in Zenker-acetic fluid, embedded in paraffin, and stained with hematoxylin eosin.

At autopsy the weight of the experimental animals averaged 3.7 gms. (12.5 percent) less than the controls. The area of the skin where DDT in kerosene had been applied showed epilation and some thickening in all animals, and in two animals there was patchy ulceration. No tumors were found in the painted area by either gross or microscopic examination. The weights and gross appearance of the liver, kidney, and other organs of the experimental animals could not be distinguished from those of the controls.

Microscopically the skin from the painted area showed several changes. There was marked atrophy of hair follicles, which were reduced from ⅛ to ¼ of the normal number. An inflammatory reaction of the dermis with an infiltration of lymphocytes, plasma cells, monocytes, and occasional polymorphonuclear leukocytes was seen. There was also some edema of the superficial portions, suggesting that an acute and chronic inflammatory response had developed in the painted area.

In addition to these general changes in the dermis of all the painted animals, the epidermis showed focal changes at the site of painting. The epidermis varied considerably in thickness. As compared to the normal in which the epithelial layer was 3–4 cells thick, in some of the treated areas of the skin the epidermis was as thin as 2 cells while in others it was 10 cells thick. This thickening affected all layers of the skin: the stratum corneum, the stratum granulosum, and the stratum malpighium. Varying degrees of disturbance of keratinization of individual epithelial cells were seen in the different layers. In three animals the prickle cell layer was increased in thickness, chiefly as a result of enlargement of cells. While ulceration of the skin was seen in only 2 cases, in 6 animals several small abscesses were seen in the epidermal layer consisting of collections of polymorphonuclear leukocytes and necrosis of epithelium with edema of the prickle-cell layer. In some, this lesion was located at the opening of the hair follicles, while in the majority it did not have this distribution. No increase in mitotic figures, abnormal cells, or failure of differentiation of the epithelial cells was seen.

The livers of all the experimental animals showed varying degree of disturbances of architecture. Considerable variation in the size of the liver cells and the nuclei was seen. There was vacuolation of the liver cells. The nuclei in many of these cells contained inclusions consisting of acidophilic material and clear spaces. Some of the nuclei were hyperchromatic. Similar changes, perhaps to a lesser extent, were seen in about ¼ of the controls.

The livers of both the experimental and control animals showed small foci of leukocytic infiltration. In the experimental animals this lesion

was accompanied by hyalinization and actual necrosis of liver cells, whereas in the control group no necrosis was seen, and there was hyalinization of liver cells in only one case. In order to compare the process in the experimental animals with that seen in the controls, it may be stated that in the former group one animal showed no necrotic area per section, in 4 animals there were 1 to 4 such foci, and in 8 there were 5 to 10, while in one there were many.

In the controls only 1 animal, which showed many foci of leukocytic infiltration, also showed some hyalinization of liver cells, but there was no necrosis. No leukocytic infiltration was seen in 3 animals, 4 such foci were seen in 7 animals, and from 5 to 10 foci in 4 animals.

Adenomatous lung tumors were seen in five animals of the experimental group. Three animals showed bronchial and lobar pneumonia. Six animals of the control group showed adenomatous lung tumors that were somewhat larger and more conspicuous than those in the experimental animals.

In comparison with the controls, no alterations could be seen in the other organs of the experimental animals.

COMMENT

The data presented above indicate that strain C mice did not develop neoplastic lesions in response to 52 weekly paintings with 5 percent DDT in kerosene. It may be noted that this exposure covered more than half the life expectancy of the test animals (*10*). This strain, moreover, has demonstrated its susceptibility to carcinogens applied by skin painting (*11*) and to those given subcutaneously (*12*) and intravenously (*13*) as well. The latent periods for tumors thus produced were 4 to 8 months, a figure well within the exposure period used in this work.

Since the mice were autopsied after a year of painting, it was not possible to observe the natural history and end result of the lesions described. However, the changes observed in the skin, including atrophy of hair follicles, hyperkeratosis, parakeratosis, acanthosis, and acute and chronic inflammation and fibrosis of the dermis, are not considered precancerous. They may have been produced by the kerosene, which is a primary skin irritant (*14*).

The liver changes observed may not be a specific result of the painting, since the focal necrosis, inflammatory cell infiltration, and the hepatic cellular and nuclear changes, though more prominent and widespread in the painted animals, were also observed in the unpainted controls. Similar hepatic cellular and nuclear changes in strain C mice have been described by Andervont and Dunn (*15*).

In their long-term study of rats fed DDT, Fitzhugh and Nelson (*7*) observed liver damage in all exposed animals and noted that of 75 rats fed DDT for 18 months or longer, 4 developed "hepatic cell tumors," and 11 showed varying amounts of "nodular adenomatoid hyperplasia." They state that about 1 percent of their older rats spontaneously developed

hepatic-cell tumors and suggest that DDT has a "distinct although minimal tumorigenic tendency."

Although it is possible that the painted mice might have developed liver tumors if they had been allowed to live longer, the degree of liver damage did not appear to be of the same order of magnitude as that which apparently preceded the development of hepatic tumors in rats fed DDT.

The incidence of lung tumors in the experimental and control mice was almost identical, *i. e.*, 36 percent in the experimentals and 38 percent in the controls. An incidence of 23 percent spontaneous pulmonary tumors in 650 breeding females was recently reported in this strain (*10*).

SUMMARY

Neither benign nor malignant tumors were observed in strain C mice painted weekly for 52 weeks with 5 percent DDT in kerosene. Histologic examination of the tissues of painted mice showed evidence of acute and chronic inflammatory changes in the painted skin and minimal liver damage.

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MUCOLYTIC ENZYME SYSTEMS. XII. HYALURONIDASE IN HUMAN AND ANIMAL TUMORS, AND FURTHER STUDIES ON THE SERUM HYALURONIDASE INHIBITOR IN HUMAN CANCER[1,2,3]

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INTRODUCTION

One aspect of the present series of investigations on hyaluronidase, and its inhibition by blood serum, is the possible role of hyaluronidase in malignant disease. It would appear that this enzyme, by virtue of its "spreading factor" properties, might enhance the invasiveness of malignant cells. Attempts to determine whether cancer tissues actually possess hyaluronidase, or whether the injected enzyme can potentiate growth or spread of tumors, have been inconclusive and often inconsistent. Some of the background literature dealing with this problem has been cited in an earlier communication (*1*) and need not be reviewed here. However, several additional references that are pertinent to this discussion are included for the sake of completeness (*2–5*).

For an understanding of the mechanism of tumor development, it is essential to know quite definitely whether cancer tissues elaborate, or at least contain, hyaluronidase. This point cannot be easily settled by use of the biologic assay, which depends on observations of the spread of an indicator, such as India ink, in rabbit skin. Aside from the lack of precision of this principle as a quantitative procedure, it is clear that false-positive results may be obtained because of the presence of concomitant substances, such as cysteine and glutathione (*6*), ascorbic acid and its oxidation products (*7*, *8*) and copper (*9*), in the tissue extracts to be assayed, since these too have been shown to lower the viscosity of hyaluronic acid. Of course, suitable controls should obviate this difficulty, but these have seldom been included.

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In some of the later work the more reliable turbidimetric or viscosimetric methods of hyaluronidase assay were used, but it is unfortunate that microbiologic sterility of the extracts of tumor tissue to be tested was not maintained. Hence, it is not certain whether the enzyme activities reported were due to the tumor material or the contaminating organisms. In fact, in a preliminary phase of this study, which was carried out with the collaboration of Dr. E. Y. Hakanson, it had been observed that both malignant, and adjacent nonmalignant, tissues possessed hyaluronidase activity, but microorganisms could be demonstrated in cultures of the material. Subsequently, details of handling the material were worked out so that sterile samples were obtained for tests of the enzyme, and the present study includes a description of this work.

The second aspect of the present investigation involved an extension of the previous study (*1*), in which it had been reported that no significant difference in the serum hyaluronidase inhibitor level was found between a group of 90 normal individuals and one of 15 cases of benign tumors, while significant elevations were observed in a series of 70 patients with nonmetastatic malignant disease, and a still greater increase in a metastatic group of 80 cases. In this study additional data were accumulated on the serum inhibitor values in cases of benign and malignant tumors, and repeated measurements on the same patient were made in some instances in order to determine what correlation might exist between the inhibitor value and the clinical condition.

MATERIALS AND METHODS

Great pains were taken to obtain nonnecrotic, clean, tumor tissue in a sterile condition. It was found that tissue from tumors of the gastrointestinal tract as well as from surface tumors of the skin and mucous membranes could not be obtained uncontaminated by microorganisms. Accordingly, tumor material of this type was not used unless the malignant lesion had extended sufficiently from its primary site, as in metastases to regional, lymph nodes, to permit resection of sterile tissue.

The tumor tissue was obtained immediately after removal at operation, freed of blood as much as possible, and then either homogenized and extracted at once, or, if this was not convenient at the time, stored at −25° C. until it could be worked up. The extract to be tested for hyaluronidase activity was prepared in sterile apparatus with sterile technique as follows: 5 gm. of tissue taken from the main tumor mass was ground in a mortar with 10 cc. of distilled water and then homogenized for 3 minutes in a Potter-Elvehjem apparatus. After standing for 1 hour at 4° C. to allow more complete extraction, the material was centrifuged at about 3000 r. p. m. for 30 minutes at 4° C. The supernatant fluid was sucked through a sintered glass filter (medium porosity) and this liquid was either tested for enzyme activity immediately or held at −25° C. for subsequent assay.

Hyaluronidase activity was measured viscosimetrically at 38° C., using hyaluronic acid prepared from human umbilical cord as described previously (*10, 11, 6*). The reaction mixture consisted of 2 cc. of the

tumor extract plus 4 cc. of veronal-buffered substrate solution of pH 6.8, and 5 cc. of the mixture were used in the viscosity tube. In a parallel control experiment distilled water was substituted for the tumor extract. By means of a stopwatch, the viscosimeter outflow times were determined initially and at intervals up to 18 hours. It was found impractical to sterilize the substrate solution; however, it was prepared from dry, heat-sterilized hyaluronic-acid powder, which was dissolved in sterile veronal-buffer solution (*6*). Any contamination that might affect the viscosity of the substrate would become apparent in the control experiment, which thus provided a check of this factor. The viscosimeter tubes were dried by repeated acetone washings before use.

For each of the tumors examined, cultures were made separately of the fresh tissue, the homogenized material, and the final extract after filtration. The culture media used in each case were Brewer's fluid thioglycollate medium, Todd-Hewitt medium, and lactose or tryptose broth. Thus, 9 cultures were set up for each tumor sample. When the positive cultures were obtained, identification of the organisms was carried out by means of routine identification media, including blood glucose agar. All cultures were examined after a 48-hour incubation period.

The measurement of the hyaluronidase inhibitor in the blood serum, and the preparation of the materials for this assay, followed the modified procedure (*11*) of the viscosimetric method first used (*10*). Hyaluronic acid prepared from human umbilical cord and hyaluronidase from bull testes were used. The results were expressed as percent inhibition which was calculated from the expression $\left(\frac{R-R_o}{R}\right)100$, where R_o is the time in seconds required to reduce the viscosity of the reaction mixture to half its initial value, and R is the corresponding time in the presence of the serum (*11*). Only 0.02 cc. of serum is required for each test.

In order to avoid complicating variables, only those patients were studied who had had no previous treatment, who were not terminal at the time, who had no recent record of a temperature over 99.6° F., and who had only a single demonstrable disease entity.

RESULTS AND DISCUSSION

It is apparent from table 1 that no appreciable decrease in outflow time, and hence no hyaluronidase activity, was observed in either the benign or malignant human tumor material in these cases. The evidence indicates that all these tissues were sterile at the time they were removed from the patient, and that the positive cultures which did result were due to subsequent contamination in handling. Thus, in tumor No. 2 gross mold contamination but no bacteria were found, and the mold proved to be identical with one that had been used in the same inoculating hood the previous day. In tumors Nos. 6 and 9, of the 9 cultures made for each, only 1 was positive in each case; and in tumor No. 10, all samples were positive on 1 medium but not on the other 2. The chance that the final extract might be contaminated is much greater than

TABLE 1.—*Decrease in viscosimeter outflow time (seconds) of hyaluronic acid substrate after 18 hours at 38° C.*[1]

Tumor type	Tumor extract	Control	Cultures		
			Brewer's fluid thioglycollate medium	Todd-Hewitt medium	Lactose or tryptose broth
BENIGN, HUMAN					
1. Lymphangioma of pancreas	0	1.5	(—)	(—)	(—)
2. Lipoma	1.0	1.5	Mold in all.	Mold in all.	Mold in all.
3. Lipoma	0	0	(—)	(—)	(—)
4. Thyroid adenoma	1.0	1.0	(—)	(—)	(—)
MALIGNANT, HUMAN					
5. Fibrosarcoma (no metastasis)	1.0	.5	(—)	(—)	(—)
6. Breast carcinoma (metastasized).	.5	.5	(—)	(—)	Only extract (+) (Tryptose) for coag. pos. hemolyt. staph.
7. Mixed tumor of oropharynx (metastasized).	0	1.0	(—)	(—)	(—)
8. Lung squam. cell carcinoma (metastasized).	1.0	1.5	(—)	(—)	(—)
9. Carcinomatous mixed tumor of testis (metastasized).	0	0	(—)	(—)	Only extract (+) (Tryptose) for alpha and beta hemolyt. strep.
10. Carcinomatous mixed tumor of testis (metastasized).	1.0	0	(—)	All (+) for alpha and beta hemolyt. strep.	(—)
MALIGNANT, MOUSE[2]					
11. Breast adenocarcinoma	1.0	1.5	(—)	(—)	(—)
12. Breast adenocarcinoma	.5	1.5	(—)	(—)	(—)
13. Breast adenocarcinoma	1.0	.5	All (+) for coag. pos. hemolyt. staph.	All (+) for coag. pos. hemolyt. staph.	All (+) for coag. pos. hemolyt. staph.
14. Breast adenocarcinoma	1.5	.5	All (+) for coag. pos. hemolyt. staph.	All (+) for coag. pos. hemolyt. staph.	All (+) for coag. pos. hemolyt. staph.
15. Breast adenocarcinoma	0	0	(—)	(—)	(—)

16. Breast adenocarcinoma	62. 0	1. 0	All (+) for coag. pos. hemolyt. staph. and alpha hemolyt. strep.	All (+) for coag. pos. hemolyt. staph. and alpha hemolyt. strep.	All (+) for coag. pos. hemolyt. staph. and alpha hemolyt. strep.
17. Breast adenocarcinoma	59. 5	1. 0	All (+) for coag. pos. hemolyt. staph. and alpha hemolyt. strep.	All (+) for coag. pos. hemolyt. staph. and alpha hemolyt. strep.	All (+) for coag. pos. hemolyt. staph. and alpha hemolyt. strep.
18. Breast adenocarcinoma	0	0	(—)	(—)	(—)
19. Breast adenocarcinoma	1. 0	0	(—)	(—)	(—)
20. Breast adenocarcinoma	50. 0	0	All (+) for coag. pos. hemolyt. staph. and beta hemolyt. strep.	All (+) for coag. pos. hemolyt. staph. and beta hemolyt. strep.	All (+) for coag. pos. hemolyt. staph. and beta hemolyt. strep.
21. Breast adenocarcinoma	55. 5	0	All (+) for coag. pos. hemolyt. staph. and beta hemolyt. strep.	All (+) for coag. pos. hemolyt. staph. and beta hemolyt. strep.	All (+) for coag. pos. hemolyt. staph. and beta hemolyt. strep.

The initial outflow times were in the range 110–120 seconds and complete hydrolysis of the substrate reduced this time to 40–45 seconds.

[1] AZ hybrid strain having transplants from A mice with spontaneous breast tumors. The A mice originally came from the colony of Dr. J. J. Bittner.

is contamination of the tumor tissue or homogenate, since it requires approximately 2 hours additional processing. The degree of contamination, when it did occur in these cases, was minimal and it did not confer measurable hyaluronidase activity. In contrast to this, the preliminary study carried out with the collaboration of Dr. E. Y. Hakanson showed significant enzyme action in some 25 malignant human tumors and in as many samples of normal tissues that were contaminated with bacteria.

Data are also given in table 1 of experiments on adenocarcinomas of the breast in 11 mice. It may be seen that in each case either all of the cultures were positive or they were all negative. Since these tumors were transplants, it is likely that those showing contamination had been infected prior to removal of the tissue for this study. It was noted that the growth was much more abundant in these positive cultures than in those from the human material included in table 1. The 5 mouse-tumor preparations that were sterile had no demonstrable hyaluronidase activity, and of the 6 contaminated cases the enzyme was found in 4.

The data in table 1 would indicate that neither benign nor malignant tumor tissues have any hyaluronidase activity unless microorganisms containing this enzyme are present.

The results of a study of the hyaluronidase inhibitor in the blood serum of patients with benign tumors are given in table 2, and in confirmation of the earlier paper (*1*) the values obtained were essentially normal as seen in table 4.

TABLE 2.—*Percent inhibition of hyaluronidase by serum of patients with benign lesions*

Site and type of lesion	Number of cases	Mean	Site and type of lesion	Number of cases	Mean
Breast [1]	10	24	Chronic stasis ulcers	3	20
Leukoplakia of tongue	1	30	Chronic duodenal ulcers	7	27
Lipoma	2	28	Xeroderma pigmentosa	1	21
Granuloma of ear	1	28			

[1] Including fibromas, fibrocystic disease, gynecomastia, and intraductal papillomas.

The data on the serum inhibitor in patients with malignant tumors are listed in table 3, and summarized in table 4. The inhibitor levels in the nonmetastasized cases were essentially normal. This would appear to be at variance with the data previously reported (*1*), in which elevations above the normal were found. However, it should be pointed out that in the present cases the majority of the lesions were small and localized, and in this type of nonmetastasized tumors normal values had also been found in the earlier study. When obvious metastases had occurred, significant elevations in the inhibitor level were observed, and this finding is in accord with the previous work (*1*). Statistical analysis of the data revealed that the difference between the metastatic malignant group and any of the others was highly significant ($p = < 0.001$).

TABLE 3.—*Percent inhibition of hyaluronidase by serum of patients with malignant tumors*

Primary site of tumor	Non-metastasized		With metastases	
	Number of cases	Mean	Number of cases	Mean
Breast adeno or scirrhous carcinoma	4	25	11	33
Oral and lip carcinoma	5	21	5	39
Stomach carcinoma			5	36
Skin basal and squamous cell carcinoma	7	20	1	40
Malignant melanoma	2	15	1	47
Malignant myxoma	1	24		
Rhabdomyosarcoma	1	37		
Bronchogenic carcinoma	1	15		
Parotid mixed tumor	2	12		
Prostatic carcinoma	1	33		
Lymphoblastoma			3	57
Rectal adenocarcinoma	2	23		
Hypernephroma			1	60
Osteogenic sarcoma			3	33
Thyroid carcinoma	1	17		
Myosarcoma	1	28		
Synovioma			1	38
Testis carcinomatous mixed tumor	1	30		
Carcinoma esophagus			1	33
Neurofibromatosis with malignant degeneration	1	33		

TABLE 4.—*Summary of the mean values of percent inhibition by normal benign, and cancerous sera*

Group	Number of cases	Mean	Standard error of mean
Normal	75	21	±1.0
Benign	25	23	±1.2
Malignant (non-metastatic)	30	22	±1.2
Malignant (with metastases)	32	36	±1.0

An attempt was made to correlate the height of the hyaluronidase inhibitor level with clinical changes of the patient. With this in mind, repeated analyses were carried out on the same patient as the disease progressed, but no consistent, direct parallelism was noted beyond the fact that an abnormally high value was associated with the metastatic cancer condition.

SUMMARY

Data has been presented of hyaluronidase measurements on benign and malignant human tumors and on adenocarcinomas of the breast in mice. The evidence indicates that malignant tissue contains no hyaluronidase unless it is contributed by micro-organisms, and that previous claims for the presence of the enzyme were probably based on experiments in which bacterial contamination had occurred.

Studies on the hyaluronidase inhibitor levels in the blood sera of patients revealed that normal values maintained in those with benign tumors and

in those with nonmetastasized malignant lesions that were usually well localized. A significant elevation in the inhibitor level was found in cases of metastasized malignant tumors. These data confirm our earlier findings in this respect. No direct correlation could be drawn between the serum inhibitor level and the clinical condition in human beings beyond the general elevation noted when metastasized tumors were present.

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ABSTRACTS OF ARTICLES PUBLISHED BY MEMBERS OF THE STAFF OF THE NATIONAL CANCER INSTITUTE

THE ELEVATION OF PLASMA RIBOFLAVIN IN ESTROGEN TREATED FEMALE CHICKS. *Roy Hertz, F. G. Dhyse* and *William W. Tullner.* Endocrinology 44: 283–286 (1949).

Earlier reports of a marked increase in plasma riboflavin in the chick after estrogen administration have been confirmed. This elevation in plasma riboflavin titer was also observed in riboflavin-deficient chicks. The muscle and liver content of riboflavin was not altered by estrogen administration in either normal or riboflavin-deficient chicks. In contrast with the riboflavin effect, the nicotinic-acid content of the blood remained unaltered after estrogen treatment. The blood riboflavin levels returned to normal within 6 days following cessation of estrogen injections.

QUANTITATIVE INTERFERENCE WITH ESTROGEN-INDUCED TISSUE GROWTH BY FOLIC ACID ANTAGONISTS. *Roy Hertz* and *William W. Tullner.* Endocrinology, 44: 278–282 (1949).

Quantitative inhibition of estrogen-induced tissue growth in the female genital tract by a number of folic acid anatagonists is demonstrated in the stilbestrol-treated chick and in the estradiol-treated ovariectomized rat. This inhibition is reversed by the administration of folic acid.

The significance of quantitative interference with hormonally induced tissue growth by an antivitamin is discussed.

ELECTRON MICROSCOPIC AND ELECTROPHORETIC STUDY OF TWO STRAINS OF TOBACCO MOSAIC VIRUS. *Herbert Kahler* and *M. W. Woods.* Arch. Biochem. 22: 393–401 (1949).

Two strains of tobacco mosaic virus, 2A a typical green strain and 1C a strain which induces intranuclear inclusions in various hosts, were examined under the electron microscope and found to be similar to the normal in size (15×280 mμ). The frequency distribution in lengths for material prepared by a chemical method did not differ appreciably from previously published distributions obtained with virus purified by centrifugation. Both strains of virus became aggregated end-end when exposed to 5-percent $(NH_4)_2SO_4$ for 2 hours at room temperature, followed by dialysis against distilled H_2O. Similar results were found using phosphate pH 6.8 to which was added 4-percent KCl. The aggregation of tobacco mosaic virus 2A was found to be more rapid at 26° C. than at 4°. Thus, no significant difference was seen in size or shape under different conditions. However, in the Tiselius electrophoretic cell, 2A was found to have a higher mobility than 1C. A pooled sample was fractionated by electrophoresis, and the two components were found, by testing in plants, to yield their characteristic lesions. The similarity in size and linear aggregation velocity between the two strains, on the one hand, suggests similar structures at the ends of the particles; on the other hand, differences in electrophoretic mobility indicate a difference in structure affecting total surface charge.

COMBINATION OF TISSUES FROM DIFFERENT SPECIES IN FLASK CULTURES. *Clifford Grobstein* and *J. S. Youngner*, Science 110: 501–503 (1949).

Using Carrel flask cultures, spleen, kidney, and lymph node explants from the mouse were grown in intimate contact with spleen, kidney, and heart explants from rat, guinea pig, or chicken. No evidence of attraction or antagonism was found even when the donor mouse was previously immunized with antigens derived from the other species used in the culture combination. It is suggested that a method is thereby provided for studying healthy cells of two species in close physiological relations.

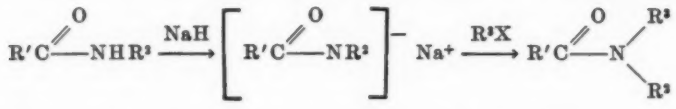
DIRECT RING-CLOSURE THROUGH A NITRO-GROUP IN CERTAIN AROMATIC COMPOUNDS WITH THE FORMATION OF NITROGEN HETEROCYCLES: A NEW REACTION. *Henry C. Waterman* and *Donald L. Vivian.* J. Organ. Chem. 14: 289–297 (1949).

A ring-closing reaction of certain aromatic nitro-groups, brought about by abstracting the nitro-group oxygen atoms under conditions preventing their replacement by hydrogen and resulting in heterocycles of which the nitro-group nitrogen atom is a member, is shown to occur in several variations, illustrated by closures of the phenazine, carbazole, benzocinnoline, benzimidazole, and other rings.

For nitro-group ring closures effected poorly or not at all by iron or lead, use of ferrous oxalate (a source of pyrophoric ferrous oxide at moderately high temperatures) is introduced. It has been possible to obtain this oxalate in an anhydrous state, despite literature assertions to the contrary, and use of this anhydrous material has been found to be advantageous.

THE USE OF SODIUM HYDRIDE IN THE ALKYLATION OF SUBSTITUTED AMIDES. *William S. Fones.* J. Organ. Chem. 14:1099–1102 (1949).

A number of substituted amides were prepared according to the general equation,



When R′ and R^2 were methyl and R^1 was aryl yields of 70–89% were obtained except where R^2 was p-nitrophenyl in which case the reaction failed.

When R′ was methyl and R^2 was phenyl changes in R^1 from primary to allyl to secondary led to yields of 79%, 73%, and 53% respectively. Using the same R′ and R^2 groupings no product was obtained when R^1 was tertiary.

If both R′ and R^2 were phenyl and R^1 was methyl a 62% yield of methyl benzanilide resulted.

In the two runs tried where R′ and R^2 were alkyl the yield of dialkyl amide was slightly lower than when R^2 was aryl.

During the experiments two new compounds, allylacetanilide and N-acetyl, N-methyl, p-aminoazobenzene were prepared and characterized.

METHYL-BIS (BETA-CHLOROETHYL)AMINE IN LARGE DOSES IN THE TREATMENT OF NEOPLASTIC DISEASES. *H. R. Bierman, M. B. Shimkin, J. Weaver, Wilbur C. Berry, and Samuel P. Wise.* California Medicine 71: 117–125 (1949)

Sixty-seven patients with neoplastic diseases were treated with 151 courses of methylbis (beta-chloroethyl)amine hydrochloride (HN_2). Seventy-seven of the courses consisted of single injections of 0.2 to 0.4 mg. per kilogram of body weight,

and 35 courses were given as single injections of 0.6 mg. per kilogram of body weight.

Twenty-three patients with Hodgkin's disease were treated. Remissions averaged approximately three months in 13 patients who were in good or fair general physical condition, and 1.5 months in 11 patients who were in poor or moribund condition; one of the 11 did not respond to the therapy.

Fifteen patients with lymphosarcoma were treated. Remissions averaged between one and two months in four patients who were in good or fair general physical condition. Of the remaining 11 patients, two showed no response, and the longest remission among the remaining nine was approximately 40 days.

Satisfactory remissions of one to three months were obtained in four patients with mycosis fungoides treated with single courses of 0.3 mg. per kilogram of body weight.

Serious toxic reactions were observed in six patients, four of whom died. In five of the six instances the reactions consisted of pancytopenia and hemorrhagic diathesis. All these patients were in poor general or hematologic status before therapy.

In general, large single doses of HN_2 were neither more nor less effective than the 4 to 6 day course usually employed with this agent. Combination of the administration of HN_2 with artificial hyperpyrexia, or with concurrent courses of pteroylglutamic conjugates, did not enhance the therapeutic effects of the agent.

ANNOUNCEMENT

National Research Council Symposium of Chemical-Biological Coordination Center

The National Research Council's Chemical-Biological Coordination Center will hold a two-day *symposium on chemical-biological correlation* in Washington on May 26–27. The meeting will include papers on the relation of structure of compounds to their biological activities, panel discussions, and a session illustrating the operations of the Center and the services it can provide to scientists.

Papers scheduled for the first session are: "The Toxicity and Parasiticidal Action of Aromatic Arsenicals in Relation to their Structure," Harry Eagle, National Institutes of Health, and G. O. Doak, University of North Carolina; "Structure—Activity Relationships in Plant Growth Regulators," A. Geoffrey Norman and Robert L. Weintraub, Chemical Corps; and "Review of the Structural Requirements for Sympathomimetic Drug Action," Alonzo M. Lands, Sterling-Winthrop Research Institute.

The second session, explaining the work of the Chemical-Biological Coordination Center, will include talks on the chemical and biological codes developed by the Center for its punched-card files, the Center's screening services, and discussions on methods of handling typical questions which come to the Center. David F. Marsh, University of West Virginia, and George Anderson, American Cyanamid Company, will compare the relationship between structure and activity of antihistaminic and antithyroid compounds, respectively, as indicated by the Center's punched-card system, with conclusions derived from their previous studies.

A panel discussion on antimalarials is scheduled for the evening session of the first day of the meeting. Robert C. Elderfield, Columbia University, will be the moderator.

The first session of the second day will hear papers on: "The Relation of Structures of Diphenyl Derivatives to their Fungicidal Action," James G. Horsfall, Connecticut Agricultural Experiment Station; "Structural Changes in Plant Insecticides as Related to Insect Toxicity," H. L. Haller, U. S. Dept. of Agriculture; and "Effect of Drugs on Physiologically Active Thiol Systems," Maxwell Schubert, New York University College of Medicine.

At the last session of the symposium, Harris L. Friedman, Lakeside Laboratories, will present a paper on "The Influence of Isosteric Replacements on Biological Activity." A combined panel discussion on antimetabolites, carcinogenesis, and cancer chemotherapy will follow; M. J. Shear, National Institutes of Health, will be the moderator.

Names of the members of both the panel on antimalarials and the combined panel on antimetabolites and cancer will be announced later. It is expected that a symposium volume will be prepared and published after the meeting. Those interested in attending should register as soon as possible with the Symposium Committee, Chemical-Biological Coordination Center, National Research Council, 2101 Constitution Avenue NW., Washington 25, D. C.

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